

II. REMARKS

Claims 1 to 20 are pending in the subject application. Claims 1-4, 6-14, and 16-20, are withdrawn from examination as being drawn to non-elected inventions pursuant to 35 U.S.C. § 121. Claims 5 and 15 are currently under examination.

By this Amendment and Response, claim 5 has been amended without prejudice or disclaimer to recite that the FRP-4 protein is a mammalian frizzled related protein 4 ("FRP-4"). The amendment to claim 5 is not intended to be a dedication to the public of the subject matter of the claim as originally filed. Applicants reserve the right to file the same or similar claim in a continuation application.

Support for the amendment to claim 5 is found in the specification on page 9, lines 12 and 13. Accordingly, an issue of new matter is not raised by this amendment and entry thereof is respectfully requested.

The specification also has been amended to remove embedded hyperlinks and/or other forms of browser-executable code. An issue of new matter is not raised by these amendments.

In view of the preceding amendments and remarks that follow, reconsideration and withdrawal of the objections and rejections are respectfully requested.

Objection to the Specification

The specification was objected to because it contains embedded hyperlinks and/or other forms of browser-executable code. The Office noted that attempts to incorporate subject matter into the patent application by reference to a hyperlink and/or other forms of browser-executable code is considered to be an improper incorporation by reference.

In response to the Office's objection, the specification has been amended to remove references to hyperlinks and/or other forms of browser-executable code. In view of these amendments, reconsideration and withdrawal of the objection to the specification is respectfully requested.

35 U.S.C. § 112, Second Paragraph

The specification was objected to, and claims 5 and 15 stand rejected under 35 U.S.C. § 112, second paragraph, for allegedly being indefinite for use of the abbreviation “FRP-4” in claims 5 and 15. In response to the Office’s rejection, claims 5 and 15 have been amended to incorporate the name of the protein in its complete form. Support for this amendment is found throughout the specification, for example on page 1, lines 8 and 9; page 5, lines 12-17; and page 7, lines 5-9. In view of this amendment, reconsideration and withdrawal of the objection to the specification and rejection of claims 5 and 15 under 35 U.S.C. § 112, second paragraph, is respectfully requested.

35 U.S.C. § 112, First Paragraph

Claim 5 stands rejected under 35 U.S.C. § 112, first paragraph, for allegedly containing subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor(s), at the time the application was filed, had possession of the claimed invention.

The Office alleged that claims 5 and 15 read on any FRP-4 protein, including sequences from any species, mutated sequences, polymorphic and allelic variants, splice variants, sequences that have an unspecified degree of identity (similarity, homology), and so forth. The Office opined that the specification as filed provides only a description of human frizzled-related protein, FRP-4, polynucleotide and amino acid sequences (see SEQ ID NOs. 1 and 2 (Figures 1 and 2), respectively), but does not provide sufficient description that would allow one of skill in the art to use SEQ ID Nos. 1 and 2 to predict the structures of any FRP-4 protein isolated from other sources, including all polymorphic, allelic and variants of this protein.

Applicants respectfully traverse. Without conceding correctness of the Office’s position, claims 5 and 15 are amended to the use of a mammalian FRP-4 protein. Support for this amendment is found on page 9, lines 12 and 13 and page 15, lines 29 to 31, of the specification. The specification enables the making and using of these proteins because the specification as filed discloses the complete amino acid sequence of human FRP-4 protein and the amino acid

sequences of murine and rodent were publicly available at the time the application was filed from journal articles and in gene and protein databases. Applicants' have attached for the Office's convenience the results of a search on the GenBank database for all proteins and nucleic acids known for FRP-4. As the Office will note, human, murine and rodent sequences were publicly available at the time the application to which the subject application claims the benefit of, was filed.

In support of their position, Applicants note that the law does not require an applicant for a patent to provide in the specification information well known in the art. (See, e.g., In re Buchner, 929 F.2d 660, 661, 18 U.S.P.Q.2d 1331, 1332 (Fed. Cir. 1991)). Thus, Applicants' specification and the publicly available information fully enables the scope of the claims.

Claims 5 and 15 also stand rejected under 35 U.S.C. § 112, first paragraph, for allegedly failing to enable one skilled in the art to which it pertains, or with which it is most nearly connected, to make and/or use the invention. The Office alleges that, in view of statements found in the literature and the general unpredictability in the art, that the specification does not provide reasonable expectation that the proteins will perform the object of the claim when administered *in vivo*. The Office concluded that therefore the quantity of experimentation required to practice the invention would include the *de novo* determination of how to engineer and deliver a FRP-4 protein such that phosphate re-absorption would be reduced in a subject, particularly, in view of the obstacles needed to overcome to use FRP-4 protein therapy.

Applicants respectfully traverse and maintain that the scope of the amended claims are fully supported by the specification as filed and information available to the public at the time the application (and the application to which the subject application claims the benefit of) was filed.

The law does not require a specification to provide working examples of all embodiments encompassed by the claims, nor is it required that any one embodiment have been actually reduced to practice for satisfaction of the enablement requirement of 35 U.S.C. § 112, first paragraph. In re Wright, 999 F.2d 1557, 27 U.S.P.Q.2d 1510 (Fed. Cir. 1993); In re Long, 368 F.2d 892, 151 U.S.P.Q. 640 (C.C.P.A. 1960).

Applicants' specification enables one to make and use the invention of the claims, see for example page 9, lines 7-13; page 12, lines 20-22; and page 23, lines 1-11 and 22-32. The specification clearly sets forth how to reduce the invention of the claims to practice, without an undue amount of specification. In support of their position, Applicants attach hereto a copy of their manuscript accepted for publication which discloses data showing that FRP-4 protein infusions specifically increased renal fractional excretion of inorganic phosphate and decreased serum phosphate. Thus, FRP-4 was shown by Applicants to reduce phosphate re-absorption *in vivo*, after administration *in vivo*, consistent with the teachings of the specification as filed.

In view of the preceding amendments, remarks, and attached manuscript, Applicants have overcome the rejections of the claims under 35 U.S.C. § 112, first paragraph and removal of these rejections are respectfully requested.

III. CONCLUSION

No additional fee is deemed necessary in connection with the filing of this Response. However, if the Patent Office determines that an extension and/or other relief is required, Applicants petition for any required relief including extensions of time and authorizes the Commissioner to charge the cost of such petitions and/or other fees due in connection with the filing of this document to **Deposit Account No. 50-2518**, referencing billing number **7009072002**. However, the Commissioner is not authorized to charge the cost of the issue fee to the Deposit Account.

Should a telephone advance prosecution of the subject application, the Examiner is invited to contact the undersigned at (650) 849-4950.

Respectfully submitted,

Date: June 20, 2003

By: 
Antoinette F. Konski
Reg. No. 34,202

Bingham McCutchen LLP
Three Embarcadero Center, Suite 1800
San Francisco, CA 94111
Telephone: (650) 849-4950
Facsimile: (650) 849-4800

Secreted Frizzled-Related Protein 4 is a Potent Tumor-derived Phosphaturic Agent

Theresa Berndt^{1,2}, Theodore A. Craig^{1,2}, Ann E. Bowe⁵, John Vassiliadis⁵, David Reczek⁵, Richard Finnegan⁵, Suzanne M. Jan De Beur⁴, Susan C. Schiavi^{5,6} and Rajiv Kumar^{1, 2, 3}

¹Department of Medicine, Nephrology Research Unit, ²Mayo Proteomics Research Center, and ³Biochemistry and Molecular Biology, Mayo Clinic and Foundation, Rochester, MN 55905

⁴Department of Medicine, Division of Endocrinology, Johns Hopkins University, Baltimore, MD 21287

⁵Receptor Ligand Therapeutics ⁶Renal Sciences Group, Genzyme Corporation, One Mountain Road, Framingham, MA 01701

Address inquiries to:

Rajiv Kumar
911A Guggenheim
Mayo Clinic and Foundation
200 First St SW
Rochester, MN, 55905
Phone: (507) 284-0020
Fax: (507) 266-4710
E-mail: rkumar@mayo.edu

or

Susan Schiavi
Genzyme Corporation
One Mountain Road
Framingham, MA, 01701
Phone: (508) 270-2354
Fax: (508) 271-2671
E-mail: susan.schiavi@genzyme.com

Supported in part by NIH grants DK25409, DK58546 and DK59505 and a grant from Genzyme Corp (to RK).

Abstract:

Tumors associated with osteomalacia elaborate a novel factor(s), “phosphatonin(s)”, which causes phosphaturia and hypophosphatemia by cAMP-independent pathways. We show that secreted frizzled-related protein-4 (sFRP-4), a protein highly expressed in such tumors, is a circulating phosphaturic factor that antagonizes renal Wnt-signaling. In cultured opossum renal epithelial cells, sFRP-4 specifically inhibited sodium-dependent phosphate transport. sFRP-4 infusions in normal rats over 2h specifically increased renal fractional excretion of inorganic phosphate (FE_{Pi}) from $14\pm2\%$ to $34\pm5\%$ (mean \pm SEM, $p<0.01$). Urinary cAMP and calcium excretion were unchanged. In thyro-parathyroidectomized rats, sFRP-4 increased FE_{Pi} from $0.7\pm0.2\%$ to $3.8\pm1.2\%$ ($p<0.05$), demonstrating that sFRP-4 inhibits renal inorganic phosphate reabsorption by parathyroid hormone-independent mechanisms. Administration of sFRP-4 to intact rats over 8h increased FE_{Pi} , decreased serum phosphate (1.95 ± 0.1 to 1.53 ± 0.09 mmol/L, $p<0.05$) but did not alter serum 1α , 25-dihydroxyvitamin D, renal 25-hydroxyvitamin D 1α -hydroxylase cytochrome P450 and sodium-phosphate co-transporter mRNA concentrations. Infusion of sFRP-4 antagonizes Wnt action as demonstrated by reduced renal β -catenin and increased phosphorylated β -catenin concentrations. sFRP-4 is detectable in normal human serum and in the serum of a patient with tumor-induced osteomalacia. Thus, sFRP-4 displays “phosphatonin” like properties, as it is a circulating protein that promotes phosphaturia and hypophosphatemia, and blunts compensatory increases in 1α , 25-dihydroxyvitamin D.

Key words

Secreted frizzled-related protein-4 (sFRP-4), phosphate, cAMP (cyclic adenosine monophosphate), 1α , 25-dihydroxyvitamin D₃, parathyroid hormone, fractional excretion of phosphate, Wnt.

Introduction:

Tumor induced osteomalacia (TIO) is a rare syndrome associated with hypophosphatemia, excessive renal phosphate excretion, osteomalacia and abnormal vitamin D metabolism (1-8). Tumors associated with this syndrome are usually of mesenchymal origin and are believed to elaborate a circulating factor known as “phosphatonin”, which is responsible for the syndrome (1-8). Complete removal of such tumors is associated with remission of the biochemical and skeletal abnormalities. In contrast to hyperparathyroidism and humoral hypercalcemia of malignancy, serum calcium, parathyroid hormone (PTH) and parathyroid hormone related protein (PTHrP) concentrations are generally normal in TIO (2-5). Serum 1α , 25-dihydroxyvitamin D concentrations, which would be expected to be increased in the presence of hypophosphatemia, are normal or reduced (2-5).

Previously, we showed that a tumor associated with this syndrome secreted a factor (or factors) that had biological properties distinct from those of other known phosphaturic proteins such as PTH and PTHrP (2). Like PTH and PTHrP, tumor supernatants inhibited sodium-dependent phosphate transport, but not sodium-dependent glucose or amino acid transport, in cultured opossum kidney (OK) cells. In contrast to the actions of PTH and PTHrP, which are mediated by 3', 5' cyclic adenosine monophosphate (cAMP), tumor cell supernatants inhibited sodium-dependent phosphate transport without altering cAMP concentrations. The inhibitory effect of tumor supernatants on sodium-dependent phosphate transport was not blocked following treatment with a PTH receptor antagonist

further indicating that the substance present in tumor supernatants was not PTH or PTHrP. This factor was named “phosphatonin” (9) to distinguish it from other known phosphaturic proteins. These findings have been subsequently confirmed by other investigators (10, 11).

Until recently, the chemical identity of “phosphatonin” has been elusive. Work by several groups demonstrated that fibroblast growth factor 23 (FGF23) is expressed in tumors associated with TIO (12-15). We, and subsequently, others demonstrated that FGF-23 specifically inhibited phosphate transport *in vitro* (12, 16). Furthermore, FGF-23 administration or over-expression in animals reproduces the renal phosphate wasting, and osteomalacia observed in TIO patients (16-18). The recent demonstration that some patients with TIO have elevated serum FGF-23 levels (19, 20) further supports the hypothesis that FGF-23 is a “phosphatonin”.

Coincident with the above studies, we performed serial analysis of gene expression (SAGE) of four tumors associated with renal phosphate wasting to identify the most highly and differentially expressed genes present in such tumors (21). In addition to *FGF-23*, we identified several other genes, including *secreted frizzled-related protein 4* (*sFRP-4*), as “phosphatonin” candidates. Given that sFRP-4 is a highly and differentially expressed, secreted protein with a previously unknown function, we investigated its potential as a “phosphatonin”.

To test whether sFRP-4 inhibits renal sodium-dependent phosphate transport, we monitored the effect of recombinant sFRP-4 on radiolabeled inorganic phosphorus uptake in renal epithelial cells *in vitro*, and we intravenously infused recombinant sFRP-4 in rats and mice to assess its affect on solute transport *in vivo*. We now report that sFRP-4 is a potent phosphate-regulating agent *in vitro* and *in vivo*, and that it acts independently of PTH. Furthermore, sFRP-4 disrupts the compensatory increase in renal 25-hydroxyvitamin D 1 α -hydroxylase cytochrome P450 messenger RNA concentrations and serum 1 α , 25-dihydroxyvitamin D concentrations typically observed in response to hypophosphatemia. To determine whether sFRP-4 is indeed a circulating factor as is characteristic of “phosphatonin”, we measured and detected sFRP-4 in normal human serum and in the serum of a patient with TIO. Moreover, we demonstrate that sFRP-4 functions as an antagonist of Wnt-signaling in the kidney as the administration of sFRP-4 *in vivo* is associated with a decrease in total β -catenin and an increase in phosphorylated β -catenin in renal tissues. Our data show that sFRP-4 could potentially function as a “phosphatonin”. Thus, tumors associated with osteomalacia elaborate at least two phosphaturic factors, FGF-23 and sFRP-4.

Methods and Materials:

Synthesis and Purification of Secreted Frizzled-Related Protein-4: A cDNA pool was synthesized from a TIO tumor using the Lambda ZAP-CMV cDNA synthesis kit (Stratagene, La Jolla, CA) (22, 23). Full-length human *sFRP-4* cDNA containing the open reading frame minus the stop codon was amplified from the cDNA pool using the sense primer 5'GCAGTGCCATGTTCTCTCCATCC3' and the antisense primer 5'CACTCTTTTCGGGTTTGTTC3' and high fidelity *Taq* DNA polymerase (Invitrogen Corp., Carlsbad, CA) (22, 23). The amplicon was cloned in frame with the V5-His epitopes into pcDNA3.1-V5-His/TOPO (Invitrogen Corp., Carlsbad, CA) or pIB/V5-His insect vector (Invitrogen Corp., Carlsbad, CA) (22, 23) and sequence fidelity confirmed (Sequagen, Worcester, MA). BTI-TN-5B1-4 (High Five™) *Trichoplusia ni* insect cells were stably transformed with pIB/V5-His-sFRP-4-V5his6X and grown in Express Five serum-free medium supplemented with 90 ml of 200 mM L-Glutamine/liter (Invitrogen Corp.) and blasticidin S for selection and maintenance of stock cultures, in 75 cm² bottles (InvivoGen, San Diego, CA). For large-scale expression of sFRP-4, a 2-L Ehrlenmeyer flask containing 0.5 L Express Five medium was inoculated with ~0.5-1 x 10⁶ cells/ml and grown at 27° C and 120 rpm in an Innova 4330 temperature-controlled incubator-shaker (New Brunswick Scientific Co., Edison, NJ). Conditioned media was obtained after 5-6 days of culture growth (final cell density ~0.4-1 x 10⁷ cells/ml). Secreted FRP-4 was adsorbed from filtered conditioned medium to 25 ml SP Sepharose resin (Amersham Biosciences, Piscataway, NJ) for ~12 hr at 4° C with stirring. The resin was batch washed sequentially, four times with each buffer using 10 volumes of 20 mM Na₂HPO₄ (pH 7.4), 65 mM NaCl (start buffer), followed by 20 mM Na₂HPO₄ (pH 7.4),

200 mM NaCl (intermediate buffer). Secreted FRP-4 was eluted with three washes of 50 ml of 20 mM Na₂HPO₄ (pH 7.4), 1 M NaCl. The eluate was dialyzed into 500 ml start buffer at 4 °C using Spectra/Por 7 dialysis tubing (1000 molecular weight cut-off, Spectrum Medical Industries, Laguna Hills, CA) with 3 changes of buffer. The dialysate was filtered (0.2 µm, 25 mm nylon syringe filter, Nalge Co., Rochester, NY) and applied to a Hi Prep™16/10 FF SP Sepharose column using an ÄKTA FPLC protein purification system (Amersham Biosciences). Secreted FRP-4 protein was eluted using a programmed elution gradient starting with 20 mM Na₂HPO₄ (pH 7.4), 65 mM NaCl and ending with 20 mM Na₂HPO₄ (pH 7.4), 1.0 M NaCl. Fractions containing sFRP-4 were identified by anti-V5 immunoblot analysis, concentrated using Centriprep 10 units (Amicon, Beverly, MA), and sterile filtered using 0.2 µm nylon syringe filters. Secreted FRP-4 protein had the expected N-terminal amino acid sequence (APC/XEAV).

Effect of sFRP-4 on phosphate transport in OK cells. Opossum-kidney cells were cultured in 45% Dulbecco's modified Eagle's medium, 45% F12 medium, with 10% fetal-calf serum. The cells were seeded at a density of about 5 x 10³ per well in 24-well tissue-culture plates and sodium-dependent phosphate, alanine, and glucose co-transport measured as described previously (2, 12). In brief, sFRP-4 or vehicle was added in varying amounts to growth medium to attain concentrations shown in Figure 1. For the measurement of sodium-dependent phosphate transport, 0.1 mM dibasic potassium phosphate was included in the transport medium and ³²P dibasic potassium phosphate was added to a final specific activity of 2 µCi/ml. For sodium-dependent alanine transport, 0.1 mM l-alanine and ³H-alanine were added (final specific activity, 1 µCi/ml). For

glucose transport, 0.1 mM methyl- α -glucopyranoside and methyl (α -d- [14 C] gluco) pyranoside were added (final specific activity, 0.2 μ Ci/ml). The transport of phosphate, alanine, and methyl- α -glucopyranoside were assayed separately. Each transport reaction was measured in three or four duplicate wells and each assay included control blank wells to correct for solute bound to cell surfaces, intracellular spaces, and culture dish.

Animals: All animal protocols were approved by the Institutional Animal Care and Use Committee of the Mayo Clinic. Male Sprague Dawley rats weighing 250-300 grams were purchased from Harlan Sprague Dawley (Madison, Wisconsin). They were fed a standard rodent formula containing 0.7% phosphorus and 0.5% calcium and adequate amounts of vitamin D. On the day of the acute experiments, rats were anesthetized with an intra-peritoneal injection of 100-150 mg/kg body wt of 5-sec-butyl-ethyl-2-thiobarbituric acid (Inactin: Byk-Gulden Konstanz, Hamburg, Germany). The animals were placed on a heated table to maintain body temperature between 36 and 38°C. After a tracheostomy, a PE-50 catheter was placed in the left carotid artery to monitor mean arterial blood pressure (MAP) and to collect blood samples. Another catheter was placed in the left jugular vein for intravenous infusion of 1% inulin in 0.9% NaCl and 2.25% BSA at a rate of 1% BW per hour and for drug administration. A PE 90 catheter was placed in the bladder for urine collection. The groups of animals studied were as follows:

Short-term (2 hour) infusion of vehicle (group 1, n = 7) or sFRP-4 (group 2, n = 10).

Effects on solute excretion in normal rats. After a 1.5-h recovery period, one 30-min urine clearance sample was taken (C1). Then, approximately 35 μ l of vehicle that

contained PBS and 0.1% BSA (Group 1) or sFRP-4 in vehicle (Group 2, 0.3 µg/kg/hr of sFRP-4), was added to the inulin/BSA infusion. After a 45-min stabilization period, a 60-minute urine clearance (C2) was taken from either vehicle-treated or sFRP-4 treated animals. A blood sample was taken at the midpoint of the clearance period. At the end of the experiment, the kidneys were removed for isolation of total RNA and quantitation of 25-hydroxyvitamin D 1 α -hydroxylase and 25-hydroxyvitamin D 24-hydroxylase cytochrome P450 mRNAs.

Short-term (2-hour) infusion of vehicle (group 3, n = 6) or sFRP-4 (group 4, n = 10).

Effect on solute excretion in thyro-parathyroidectomized (TPTX) rats. These protocols are identical to those described for groups 1 and 2, except that TPTX rats were studied two hours after the completion of thyro-parathyroidectomy. Effective thyro-parathyroidectomy was confirmed by a significant reduction in the urinary fractional excretion of phosphate, increased fractional excretion of calcium and the presence of hypocalcemia.

Long-term (8-hour) infusion of vehicle (group 5, n = 5) or sFRP-4 (group 6, n = 7).

Effects on solute excretion, serum 1,25-dihydroxyvitamin D concentrations and 25-hydroxyvitamin D 1 α -hydroxylase cytochrome P450 and 25-hydroxyvitamin D 24-hydroxylase cytochrome P450 mRNA concentrations. These protocols are identical to those described for the previous groups, except that after the control collection (C1), one hour clearances were taken at 4 hours (C2) and 7 hours (C3) after initiation of the vehicle or sFRP-4 infusions. At the end of the experiment, blood was collected for measurement

of 1α , 25-dihydroxyvitamin D, and kidneys were collected for the isolation of mRNA and assessment of 25-hydroxyvitamin D 1α -hydroxylase cytochrome P450 and 25-hydroxyvitamin D 24-hydroxylase cytochrome P450 mRNA concentrations by quantitative real-time RT-PCR. To determine if decrements in serum phosphate were associated with appropriate increases in renal 25-hydroxyvitamin D 1α -hydroxylase cytochrome P450 mRNA concentrations, rats were maintained on a 0.1% phosphate diet (low Pi) or a 0.4% phosphate diet (normal Pi) for a period of one week, so as to bring about a modest reduction in serum phosphate equivalent to that seen following sFRP-4 infusion.

Serum and urine measurements: Serum and urinary phosphate concentrations were determined using the method of Chen *et al.* (24). Serum and urine inulin concentrations were measured using the anthrone method (25). Sodium concentrations were measured in urine using an Instrumentation Laboratory flame photometer (Instrumentation Laboratory, Wilmington, MA). Serum and urine calcium concentrations were determined by atomic absorption spectrometry (26-29). Plasma 1α , 25-dihydroxyvitamin D and 25-hydroxyvitamin D concentrations were measured by radio-immunoassay (Diasorin, Stillwater, MN). Urinary cyclic adenosine monophosphate was measured using an enzyme immunoassay (Amersham Biosciences).

Real-time PCR to assess 25-hydroxyvitamin D 1α -hydroxylase cytochrome P450 and 25-hydroxyvitamin D 24-hydroxylase cytochrome P450 Messenger RNA Concentrations in the Kidney: A Polytron (Brinkman Instruments, Westbury, NY) was used to homogenize

a lateral third of each rat kidney containing cortical tissue in 4 ml of RLT buffer (Qiagen, Valencia, CA). The homogenate was centrifuged for 3 min at 4500 x g. Total RNA was extracted from the supernatant following treatment with DNase using the RNeasy midi kit (Qiagen). RNA concentration in the samples was quantitated using UV spectroscopy at a wavelength of 260 nm. Samples containing 200 ng total RNA were prepared using the Quantitect SYBR Green RT-PCR kit (Qiagen) and real-time PCR done following the manufacturer's protocol on a PE Applied Biosystems Model 7700 sequence detector (Applied Biosystems, Foster City, CA). Primers were designed to span introns and to generate amplicons of 100-120 bp. The primer sequences are as follows: 25-hydroxyvitamin D 1 α -hydroxylase cytochrome P450 (forward, 5'GCATCCATCTCCAGTTTGTAGAC3'; reverse, 5'TGTGCCTCTTGTGCATAGTAAGA3'), 25-hydroxyvitamin D 24-hydroxylase cytochrome P450 (forward, 5'GATCACCTTTCCAAGAAGGAAGT3'; reverse, 5'AGAGAATCCACATCAAGCTGTTC3'), sodium dependent phosphate co-transporter IIa forward, 5'CTGTGCACTTG TCTTATCCTCCT; reverse 5'GGAAGTCTGTGTTGATGACCTT. Long oligonucleotide templates were designed which represented the entire amplicon sequence for each gene and dilutions of which were used to generate standard curves. All samples were interrogated in triplicate, and the averages of each normalized to GAPDH. GAPDH mRNA values did not deviate significantly across samples. Normalized relative values were assigned absolute values based on the slope and y-intercept of the relevant standard curve. All PCR products, including reverse transcriptase negative control, and no template control samples were subjected to electrophoresis on a 4% agarose gel. Only expected band sizes were detected.

Enzyme-linked Immunosorbent Assay (ELISA) for sFRP-4 in Serum:

A two-antibody sandwich ELISA was developed using monoclonal antibodies (Mab 3.1 and 3.4) against insect-derived sFRP-4 protein. These antibodies were prepared in the Mayo Clinic Core Facility using established procedures (30). Briefly, Mab 3.4 was immobilized onto a Reacti-Bind ELISA plate (Pierce, Rockford, Ill). Recombinant sFRP-4 standards in 100 μ l of PBS or 100 μ l human serum samples were added to individual wells and incubated at room temperature for 1 hour. Samples were aspirated and wells washed three times with PBS and 0.5% Tween-20. A second anti-sFRP-4 monoclonal antibody, Mab 3.1 was biotinylated with sulfo-NHS-LC-biotin (Pierce, Rockford, Ill) and applied to each well and incubated for 1 hour at room temperature. Samples were aspirated and wells washed three times with PBS and 0.5% Tween-20. Streptavidin-HRP (100 ng/ml) (Pierce, Rockford, Ill) was added to facilitate detection with 3,3',5,5'-tetramethylbenzidine substrate (Sigma, St. Louis, MO). After the addition of stop solution (4ADI, San Antonio, TX), the plate was read in SpectraMax Plus spectrophotometer (Molecular Devices, Sunnyvale, CA) at 450 nm. The concentration of sFRP-4 in each serum sample was calculated based on a standard curve generated using known concentrations of purified insect sFRP-4.

Western blotting to detect β -catenin and phosphorylated β -catenin or sFRP-4 in renal tissues: Kidneys (~ 1 g tissue) from rats infused with sFRP-4 or vehicle, as described, above were homogenized in an equal volume of buffer (50 mM tris (pH 7.8), 150 mM sodium chloride) containing a mammalian protease inhibitor mixture (Sigma Chemical

Co., catalog # P8340) and phosphatase inhibitors, okadaic acid (1 μ M) and microcystin LR (1 μ M; Sigma Chemicals, St. Louis, MO). Equal amounts of protein from control and experimental kidneys were loaded and electrophoresed on SDS-polyacrylamide gels (31). The electrophoresed proteins were transferred to polyvinylidene difluoride membranes (32), and the membranes were probed with antibodies against β -catenin or phosphorylated β -catenin (Cell Signaling Technologies, Beverly, MA). Horseradish peroxidase conjugated secondary antibodies were used to detect primary antibodies bound to either β -catenin or phosphorylated β -catenin.

For detection of sFRP-4 in renal tissues, rat renal tissue homogenates were electrophoresed on SDS-polyacrylamide gels, and the electrophoresed proteins were transferred to PVDF membranes as described above (31, 32). Monoclonal antibodies raised against sFRP-4 were used to probe PVDF membranes, and bound anti-sFRP-4 antibodies were detected using an anti-mouse IgG, horseradish peroxidase-coupled antibody.

Statistics. Values are expressed as means \pm SE. Statistical comparisons for groups 1-4 were made using a paired T-test. Comparisons between multiple clearances (Groups 5 and 6) were made using one-way ANOVA followed by the Fishers PSLD test. Statistical comparison for the phosphate uptake assay was made using an unpaired t-test. A P value <0.05 was considered to be significant.

Results:

Secreted FRP-4 specifically inhibits sodium-dependent phosphate transport in opossum kidney cells: To determine whether sFRP-4 directly inhibits sodium-dependent phosphate transport in opossum kidney cells maintained in culture, we added increasing amounts of sFRP-4 to the supernatants of OK cells maintained in culture (Figure 1). As can be seen, a dose of 250 pg/ml of sFRP-4 per well caused a statistically significant decrease in sodium dependent phosphate uptake in OK cells maintained in culture. Sodium-dependent glucose transport (sFRP-4, 10.26 ± 0.33 nmol/mg protein vs. vehicle, 9.62 ± 0.45 nmol/mg protein, $p = \text{NS}$) and sodium-dependent alanine transport (sFRP-4, 0.059 ± 0.008 nmol/mg protein vs. vehicle, 0.083 ± 0.009 nmol/mg protein. $P = \text{NS}$) did not change significantly.

Effects of short-term sFRP-4 infusion on renal solute transport, renal 25-hydroxyvitamin D 1 α -hydroxylase mRNA and 25-hydroxyvitamin D 24-hydroxylase mRNA: As shown in Figure 2, sFRP-4 infused in normal rats over a period of 2 hours caused a marked increase in the fractional excretion of phosphate (FE_{P_i}) from $14 \pm 2\%$, mean \pm SEM to $34 \pm 5\%$, mean \pm SEM, $p < 0.05$. FE_{P_i} was stable ($14 \pm 3\%$ to $18 \pm 2\%$, mean \pm SEM, $p = \text{NS}$) in vehicle infused rats. Fractional sodium excretion (FE_{Na}) increased in the sFRP-4 infused rats from $1.1 \pm 0.3\%$ to $2.7 \pm 0.5\%$ (mean \pm SEM, $p < 0.05$). In the vehicle infused group, a smaller increase in FE_{Na} was noted ($0.3 \pm 0.1\%$ to $1.2 \pm 0.3\%$, mean \pm SEM, $p < 0.05$). Fractional excretion of calcium (FE_{Ca}) did not change in either group. GFR and mean arterial pressure were stable in both groups of animals (data not shown).

Of note, urinary cAMP excretion did not change in either the sFRP-4-infused (23.6 ± 2.6 to 25.5 ± 2.6 nmol/min, $p = \text{NS}$) or vehicle-infused (24.6 ± 1.2 to 28.2 ± 3.5 nmol/min, $p = \text{NS}$) groups of rats. Serum phosphate and serum calcium concentrations did not change during the short-term infusion in the sFRP-4. Renal 25-hydroxyvitamin D 1α -hydroxylase cytochrome P450 mRNA (sFRP-4: $2.29 \times 10^{-13} \pm 1.18 \times 10^{-13}$ mol/pg RNA vs. vehicle: $4.36 \times 10^{-13} \pm 1.82 \times 10^{-13}$ mol/pg RNA, $p = \text{NS}$) and 24-hydroxylase cytochrome P450 mRNA (sFRP-4: $3.73 \times 10^{-14} \pm 1.35 \times 10^{-14}$ vs. vehicle: $1.45 \times 10^{-14} \pm 4.0 \times 10^{-15}$ mol/pg RNA, $p = \text{NS}$) concentrations were similar in the sFRP-4 and vehicle treated groups.

Parallel experiments in mice were also performed using recombinant protein derived from mammalian or insect cells. Secreted FRP-4 also induced a 3.1-fold increase in the fractional excretion of phosphate in mice ($p < 0.05$). These results confirm the actions of sFRP-4 in another species and demonstrate that the method of production of recombinant sFRP-4 (mammalian vs. insect expression systems) has no major influence on protein function.

The phosphaturic action of sFRP-4 is PTH-independent: To determine whether parathyroid hormone is necessary for the phosphaturic action of sFRP-4, we infused sFRP-4 intravenously in acutely thyro-parathyroidectomized rats. Thyro-parathyroidectomy was associated with a decrease in the basal FE_{P_i} from approximately 15% in intact animals to approximately 0.7% in thyro-parathyroidectomized animals. Basal FE_{Ca} increased from 0.5% to 1.5% and serum calcium decreased from

approximately 10.5 mg/dl to 8 mg/dl. As shown in Figure 3, sFRP-4 infusion in acutely TPTX animals was associated with a 3.5-fold increase in the FE_{P_i} ($1.0 \pm 0.3\%$ to $3.8 \pm 1.2\%$, $p < 0.05$). This 3.5-fold increase during sFRP-4 infusion was similar to that observed in intact animals. The FE_{P_i} was stable in animals receiving the vehicle. There were no changes in the fractional excretion of sodium or calcium in rats infused with sFRP-4. GFR and mean arterial pressure were stable throughout the experiment.

Long-term sFRP-4 infusion results in increased fractional excretion of phosphorus, reduced serum phosphorus concentrations, and inappropriately normal serum 1α , 25-dihydroxyvitamin D concentrations: We examined the effects of a long-term (8 hr) infusion of sFRP-4 on fractional excretion of solutes, serum inorganic phosphorus, serum $1,25$ -dihydroxyvitamin D concentrations and renal 25 -hydroxyvitamin D 1α -hydroxylase cytochrome P450 and 25 -hydroxyvitamin D 24 -hydroxylase cytochrome P450 messenger RNA concentrations. With long-term infusion of sFRP-4 (Table 1), FE_{P_i} increased from $8.5 \pm 2.6\%$ to $20.2 \pm 3.7\%$ four hours after initiation of sFRP-4 infusion and was $18.2 \pm 4.3\%$ at 8 hours. FE_{P_i} did not change in the animals infused with vehicle. There were no changes in the fractional excretion of sodium or the fractional excretion of calcium in either the sFRP-4 or vehicle groups. In the sFRP-4 infused animals, there was a decrease in serum phosphorus concentrations from 1.95 ± 1.0 mmol/l to 1.51 ± 0.11 mmol/l at 4 h and to 1.53 ± 0.09 mmol/l at 8 h. There was no change in the serum phosphorus concentrations in the animals receiving vehicle. Serum 1α , 25-dihydroxyvitamin D concentrations were not different between the sFRP-4 and vehicle infused rats (89.6 ± 26.8 pg/ml, sFRP-4 group vs. 62.4 ± 29.2 pg/ml vehicle group, mean \pm SEM, $p = \text{NS}$).

25-Hydroxyvitamin D 1 α -hydroxylase cytochrome P450 messenger mRNA concentrations were similar in the vehicle and sFRP-4 infused groups at 8 hrs ($1.41 \times 10^{-13} \pm 3.87 \times 10^{-14}$ moles/pg RNA vs. $1.37 \times 10^{-13} \pm 1.77 \times 10^{-14}$ moles/pg RNA, $p = \text{NS}$, Figure 4). 25-hydroxyvitamin D 24-hydroxylase cytochrome P450 mRNA concentrations showed a tendency to increase in sFRP-4 infused rats, but this value was not statistically significant. In phosphate-deprived rats, renal 25-hydroxyvitamin D 1 α -hydroxylase cytochrome P450 messenger mRNA concentrations were appropriately increased by approximately 3.7-fold ($8.5 \times 10^{-13} \pm 9.5 \times 10^{-14}$ moles/pg RNA in phosphate-deprived rats, $n = 6$, vs. $2.3 \times 10^{-13} \pm 8.9 \times 10^{-14}$ moles/pg RNA in normal rats, $n = 6$, $p < 0.05$, Figure 4) following an ~16% decrease in serum Pi and an ~33% decrease in urinary phosphate excretion.

The phosphaturic action of sFRP-4 is not mediated through transcriptional regulation of the sodium-dependent phosphate co-transporter: We measured sodium-dependent phosphate co-transporter mRNA concentrations in sFRP-4 treated rats and found that they did not change significantly with sFRP-4 treatment (sFRP-4, $6.93 \times 10^{-11} \pm 6.41 \times 10^{-12}$ moles/pg RNA vs. vehicle, $8.67 \times 10^{-11} \pm 9.20 \times 10^{-12}$ moles/pg RNA).

sFRP-4 is detectable in normal human serum and in the serum of a patient with TIO: We measured sFRP-4 concentrations in normal human serum obtained from five normal subjects. The mean concentration was 34.8 ± 13.3 ng/ml and the range was 5.5 to 79.8 ng/ml). In a patient with TIO on with phosphate and 1 α , 25-dihydroxyvitamin D₃ serum sFRP-4 was 11.1 ng/ml.

sFRP-4 is expressed in the rat kidneys and antagonizes Wnt-signaling: Immunoblot analysis of rat renal homogenates using a monoclonal anti-FRP-4 antibody indicates that sFRP-4 protein ($M_r \sim 50,000$) is detectable in kidney homogenates (data not shown). To determine the mechanism of action of sFRP-4 in the kidney, we measured β -catenin or phosphorylated β -catenin concentrations in sFRP-4 or vehicle treated kidneys *in vivo*. As shown in figure 5, rats infused with FRP-4 have reduced amounts of renal β -catenin protein (upper panel) and increased phosphorylated β -catenin signal (lower panel) compared to those infused with vehicle.

Discussion

Tumor-induced osteomalacia (TIO) is a paraneoplastic renal phosphate wasting disorder characterized by hypophosphatemia, phosphaturia, and inappropriately normal or low serum $1\alpha, 25$ -dihydroxyvitamin D concentrations that lead to osteomalacia or rickets, muscle weakness and general debility (1-8). In TIO, serum calcium, PTH and PTHrP concentrations are generally normal. The mesenchymal tumors that cause TIO are thought to elaborate a circulating factor that causes the metabolic abnormalities noted above (1-8). Complete removal of the tumors is associated with correction of the biochemical and skeletal abnormalities.

Earlier, we demonstrated that a tumor associated with this syndrome elaborated a novel factor(s) that was responsible for the reduced renal reabsorption of phosphate (2). Recent work suggests that fibroblast growth factor 23 (FGF-23) is a “phosphatonin”-like molecule. In addition to its expression in tumors associated with osteomalacia (13, 15, 33), FGF-23 serum levels are increased in some patients with TIO and X-linked hypophosphatemic rickets (XLH) (19), a syndrome that has phenotypic features similar to TIO (34). Elevated FGF-23 concentrations in the serum of patients with TIO diminish after removal of the tumor and a return of the clinical state to normal (19). FGF-23 reduces phosphate uptake in opossum kidney cells in culture (12, 35) and may be a substrate for the enzyme PHEX (12) that is mutated in patients with X-linked hypophosphatemic rickets (36). Shimada *et al.* showed that recombinant FGF-23 reduces serum phosphate concentrations and increases urinary phosphate losses when administered to mice (13). Furthermore, nude mice implanted with an established cell

line expressing FGF-23 develop osteomalacia. Transgenic mice over-expressing FGF-23 have hypophosphatemia and increased urinary phosphate excretion (37) and null-mutant mice lacking the *FGF-23* gene are hyperphosphatemic (38). Finally, activating mutations of FGF-23 are responsible for autosomal dominant hypophosphatemic rickets (ADHR) (17, 39, 40), which also has clinical features similar to TIO and XLH. ADHR mutations have been associated with increased FGF-23 protein stability and/or activity (16-18, 40).

Our approach to identify potential “phosphatonin” candidates was to perform a comprehensive gene expression profile in four tumors associated with phosphate wasting and to compare these profiles to those of similar mesenchymal tumors not associated with TIO. We examined several tumors associated with the syndrome of TIO for the presence of differentially expressed genes using the technique of serial analysis of gene expression (SAGE) (21, 41, 42). We identified several genes that were expressed preferentially in tumors associated with osteomalacia but not in control tissues. These included *FGF-23*, *MEPE*, *dentin matrix protein 1*, and secreted *FRP-4*. Since sFRP-4 is a secreted molecule, we hypothesized that this protein could enter the circulation and reduce phosphate reabsorption in the kidney. To test this hypothesis, we biosynthesized sFRP-4 and demonstrated it specifically inhibited sodium-dependent phosphate uptake in cultured opossum renal epithelia (Figure 1). We further showed that, the infusion of sFRP-4 into rats is associated with a 2.5-3-fold increase in the urinary excretion of phosphate (Figure 2, Table 1). In acute experiments, there is an increase in sodium excretion, which is probably secondary to the infusion itself and is independent of sFRP-4 since it is not apparent in animals infused with sFRP-4 for a period of 8 hours. Urinary fractional

excretion of calcium and urinary cAMP excretion do not change. The infusion of sFRP-4 for a period of 4-8 hours was associated with a decrease in serum phosphate concentrations. Thus, this protein is capable of causing hyperphosphaturia and hypophosphatemia *in vivo*.

To determine whether the phosphaturic effects of sFRP-4 are PTH-dependent, we infused sFRP-4 into acutely thyro-parathyroidectomized rats, and showed that there was a 3-fold increase in phosphate excretion (Figure 3). This is similar to the change in FE_{Pi} observed in the intact animal suggesting that sFRP-4 does not act via parathyroid hormone-dependent pathways. The maximal absolute increase in FE_{Pi} was less in TPTX rats than in rats with intact parathyroid glands, most likely due to enhanced phosphate reabsorption by the proximal straight tubule in the hypoparathyroid state (43, 44). Further evidence that the phosphaturic effect of sFRP-4 is not PTH-dependent comes from the lack of stimulation of urinary cyclic AMP following sFRP-4 infusion in intact rats. The effect of sFRP-4 observed in OK cells maintained in culture further suggests that sFRP-4 has direct actions on Pi transport.

Infusion of sFRP-4 produces the same defect in vitamin D metabolism observed in TIO. Despite the induction of hypophosphatemia following sFRP-4 infusion, serum 1α , 25-dihydroxyvitamin D concentrations and renal 25-hydroxyvitamin D 1α -hydroxylase cytochrome P450 messenger RNA amounts fail to increase in the predicted physiological manner (Table 1) (45-50). In contrast, phosphate deprivation in normal rats that was associated with mild hypophosphatemia resulted in a significant (3.7-fold) increase in

renal 25-hydroxyvitamin D 1 α -hydroxylase cytochrome P450 messenger RNA concentrations. This failure of the vitamin D-endocrine system to respond to hypophosphatemic stimuli is characteristic of TIO and the phenotypically related XLH and ADHR (3-5, 51).

We show that sFRP-4 circulates in the serum of normal humans, suggesting that it could potentially function as a “phosphatonin”. sFRP-4 was also detectable in the serum of a patient with TIO, who had an un-resectable tumor and was on treatment with phosphate supplementation and 1 α , 25-dihydroxyvitamin D₃. The sFRP-4 concentrations were not elevated relative to the control concentrations. It is possible that treatment of the patient with phosphate and 1 α , 25-dihydroxyvitamin D₃ could have suppressed sFRP-4 concentrations. A more comprehensive study of other patients with TIO will be needed prior to making a conclusive statement regarding the usefulness of sFRP-4 concentrations in the diagnosis of TIO. It is of interest that not all patients with TIO have elevations of FGF23 and in some instances cure of the syndrome occurs without changes in FGF23 concentrations (20).

Sodium-dependent phosphate co-transporter mRNA levels were unchanged following infusion of sFRP-4 in rats. This suggests that transcriptional regulation of sodium-phosphate co-transporter mRNA concentrations by sFRP-4 is not critical in modulating phosphate transport. Others have shown that parathyroid hormone does not alter sodium-phosphate co-transporter mRNA concentrations in the kidney but causes a re-distribution in the sodium-phosphate co-transporter protein from the luminal membrane of the

proximal tubular cell into the lysosomal compartment (52-54). Preliminary data suggests that sFRP-4 also causes a re-distribution of sodium-dependent phosphate co-transporter IIa protein in OK cells (data not shown). Further experiments will be needed to precisely define the effects of sFRP-4 on sodium-dependent phosphate co-transporter IIa protein cell biology.

Our data point to a novel function of sFRP-4 in the kidney. Secreted FRP-4 belongs to a family of secreted proteins that contain a cysteine-rich domain that is homologous to the extracellular domain of the Wnt receptors, the Frizzled proteins (55-59). Secreted FRPs modulate the activities of Wnts (60-68). The Wnt signaling pathway plays an important role in renal, bone and cardiac development (69-77). Binding of Wnts to frizzled receptors leads to activation of signals through three different pathways resulting in both transcription and non-transcriptional changes (74-78). sFRP-4 mRNA is detectable in the kidney (58, 79, 80) and these data are supported by our observations that sFRP-4 protein is also detectable in homogenates of rat kidney. We show that sFRP-4 antagonizes Wnt-signaling in the kidney. The binding of Wnts to frizzled receptors normally stabilizes intra-cellular β -catenin by preventing degradation, and decreases the phosphorylation of β -catenin (58, 81-83). Increased phosphorylation of β -catenin suggests that sFRP-4 antagonizes this pathway.

Our findings add to the list of functions attributed to sFRP-4. Secreted FRP-4 expression has been correlated with the presence of apoptosis in several tissues such as osteoarthritic cartilage, but co-localization of sFRP-4 in apoptotic cells and direct induction of

apoptosis by sFRP-4 has not been demonstrated (80, 84, 85). It is also noteworthy that elevated sFRP-4 expression is correlated with several tumor types and proliferative tissues (86, 87). Based on these observations and the diverse expression pattern of sFRP-4, it is likely that additional functions will be identified (88).

In conclusion, we have shown that a protein that is over expressed in tumors associated with renal phosphate wasting and osteomalacia is capable of specifically inhibiting sodium-dependent phosphate transport *in vitro* and selectively increasing the fractional excretion of phosphorus in a parathyroid hormone independent manner *in vivo*. We also show that there is a decrease in serum phosphate concentrations in rats administered this protein for a period of 4 to 8 hours, and that the vitamin D endocrine system fails to respond to hypophosphatemic stimuli following sFRP-4 infusion. Secreted FRP-4 could potentially function as a “phosphatonin,” and it is apparent from these studies that there are at least two phosphaturic proteins (FGF23 and sFRP-4) produced by tumors associated with renal phosphate wasting and osteomalacia.

Acknowledgements: We thank Dr. Lorraine Fitzpatrick, Mayo Clinic, for providing serum on the patient with TIO.

Figure Legends:

Figure 1: Effect of sFRP-4 on sodium-dependent phosphate uptake in opossum kidney cells maintained in culture. * $p < 0.05$.

Figure 2: Effect of the infusion of sFRP-4 on solute excretion in intact rats. Intact rats were administered sFRP-4 (■) at a dose of $0.3 \mu\text{g/kg/hr}$ (Group 2) or vehicle (□) (Group 1) by intravenous infusion over a period of two hours. C1 = equilibration period prior to the infusion of sFRP-4 or vehicle. C2 = experimental period during which sFRP-4 or vehicle was infused. Fractional excretion of inorganic phosphate, sodium and calcium were measured as described in the text. * $p < 0.05$.

Figure 3: Effect of the infusion of sFRP-4 on solute excretion in thyro-parathyroidectomized (TPTX) rats. TPTX rats were administered sFRP-4 (■) at a dose of $0.3 \mu\text{g/kg/hr}$ (Group 4) or vehicle (□) (Group 3) by intravenous infusion over a period of two hours. C1 = collection period prior to the infusion of sFRP-4 or vehicle. C2 = collection period during which sFRP-4 or vehicle was infused. Fractional excretion of inorganic phosphate, sodium and calcium were measured as described in the text. * $p < 0.05$.

Figure 4: The effect infusion of vehicle or sFRP-4 ($0.3 \mu\text{g/Kg/hr}$) for eight hours, or the effect of phosphate deprivation induced by a low phosphate diet phosphate (LPD)

compared with a normal phosphate diet (NPD), on the concentration of 25-hydroxyvitamin D 1 α -hydroxylase cytochrome P-40 mRNA concentrations in kidney (* $p<0.05$).

Figure 5: Western blots of renal homogenates obtained from rats infused with vehicle (control) or sFRP-4 for 8 hours. Antibodies against β -catenin (upper panel) or phosphorylated β -catenin (lower panel) were used to detect proteins as described in the Methods section.

Table 1. Effect of Long-Term (8h) Infusion of sFRP-4 on Renal Function in Intact Rats.

	Vehicle (n=5)			sFRP-4 (n=7)		
	C1	C2	C3	C1	C2	C3
GFR (mL/min) ± SE	3.7 ± 0.6	3.2 ± 0.4	2.6 ± 0.6	3.5 ± 0.2	2.8 ± 0.3	2.4 ± 0.5
FE _{pi} (%) ± SE	7.4 ± 2.2	6.8 ± 1.7	9.9 ± 0.4	8.5 ± 2.6	20.2* ± 3.7	18.2 ± 4.3
FE _{Na} (%) ± SE	1.6 ± 0.4	1.3 ± 0.2	0.8 ± 0.1	1.9 ± 0.6	1.8 ± 0.2	2.2 ± 1.1
FE _{Ca} (%) ± SE	0.8 ± 0.2	0.8 ± 0.2	0.8 ± 0.2	0.9 ± 0.3	0.8 ± 0.1	0.7 ± 0.3
P _{pi} (mM) ± SE	2.02 ± 0.15	2.02 ± 0.22	1.83 ± 0.18	1.95 ± 0.10	1.51* ± 0.11	1.53* ± 0.09
P _{Ca} (mg/dl) ± SE	8.0 ± 0.3	9.1 ± 0.3	8.8 ± 0.6	8.7 ± 0.7	9.1 ± 0.3	9.7 ± 0.5
MAP (mm Hg) ± SE	119 ± 6	113 ± 8	108 ± 5	124 ± 10	118 ± 7	120 ± 6
Serum 1α, 25(OH) ₂ D (pg/ml)	62.4 ± 29.2			88.9 ± 26.8		
Renal 25(OH)D 1α-hydroxylase cytochrome P450 mRNA (moles/pg)	1.41x10 ⁻¹³ ± 3.87x10 ⁻¹⁴			1.37x10 ⁻¹³ ± 1.77x10 ⁻¹⁴		
Renal 25(OH) D 24-hydroxylase cytochrome P450 mRNA (moles/pg)	1.53x10 ⁻¹⁴ ± 1.38x10 ⁻¹⁵			0.92 x10 ⁻¹⁴ ± 2.48x10 ⁻¹⁵		

GFR, glomerular filtration rate; FE_{pi}, fractional excretion of phosphate; FE_{Na}, fractional excretion of sodium; FE_{Ca}, fractional excretion of calcium; P_{pi}, plasma phosphate concentration, P_{Ca}, plasma calcium concentration; MAP, mean arterial pressure.

*Indicates a significant difference, ANOVA, p<0.05.

References

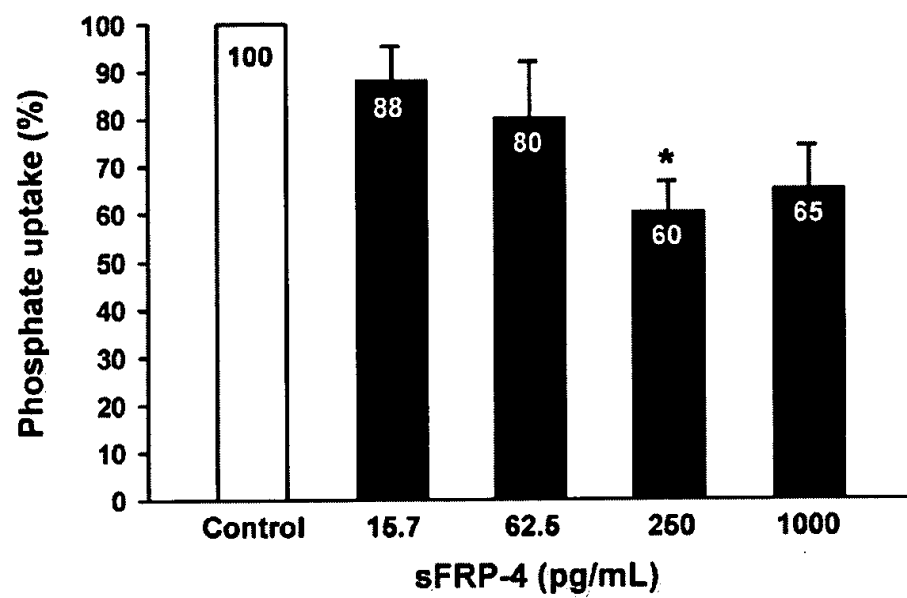
1. Aschinerberg, L.C., Solomon, L.M., Zeis, P.M., Justice, P., and Rosenthal, I.M. 1977. Vitamin D-resistant rickets associated with epidermal nevus syndrome: demonstration of a phosphaturic substance in the dermal lesions. *J Pediatr* 91:56-60.
2. Cai, Q., Hodgson, S.F., Kao, P.C., Lennon, V.A., Klee, G.G., Zinsmeister, A.R., and Kumar, R. 1994. Brief report: inhibition of renal phosphate transport by a tumor product in a patient with oncogenic osteomalacia. *N Engl J Med* 330:1645-1649.
3. Kumar, R. 2000. Tumor-induced osteomalacia and the regulation of phosphate homeostasis. *Bone* 27:333-338.
4. Kumar, R. 1997. Phosphatonin--a new phosphaturic hormone? (lessons from tumour- induced osteomalacia and X-linked hypophosphataemia). *Nephrol Dial Transplant* 12:11-13.
5. Kumar, R. 2002. New insights into phosphate homeostasis: fibroblast growth factor 23 and frizzled-related protein-4 are phosphaturic factors derived from tumors associated with osteomalacia. *Curr Opin Nephrol Hypertens* 11:547-553.
6. Schiavi, S.C., and Moe, O.W. 2002. Phosphatonins: a new class of phosphate-regulating proteins. *Curr Opin Nephrol Hypertens* 11:423-430.
7. Stone, E., Bernier, V., Rabinovich, S., and From, G.L. 1984. Oncogenic osteomalacia associated with a mesenchymal chondrosarcoma. *Clin Invest Med* 7:179-185.
8. Ryan, E.A., and Reiss, E. 1984. Oncogenous osteomalacia. Review of the world literature of 42 cases and report of two new cases. *Am J Med* 77:501-512.
9. Econs, M.J., and Drezner, M.K. 1994. Tumor-induced osteomalacia--unveiling a new hormone. *N Engl J Med* 330:1679-1681.
10. Nelson, A.E., Namkung, H.J., Patava, J., Wilkinson, M.R., Chang, A.C., Reddel, R.R., Robinson, B.G., and Mason, R.S. 1996. Characteristics of tumor cell bioactivity in oncogenic osteomalacia. *Mol Cell Endocrinol* 124:17-23.
11. Wilkins, G.E., Granleese, S., Hegele, R.G., Holden, J., Anderson, D.W., and Bondy, G.P. 1995. Oncogenic osteomalacia: evidence for a humoral phosphaturic factor. *J Clin Endocrinol Metab* 80:1628-1634.
12. Bowe, A.E., Finnegan, R., Jan de Beur, S.M., Cho, J., Levine, M.A., Kumar, R., and Schiavi, S.C. 2001. FGF-23 inhibits renal tubular phosphate transport and is a PHEX substrate. *Biochem Biophys Res Commun* 284:977-981.
13. Shimada, T., Mizutani, S., Muto, T., Yoneya, T., Hino, R., Takeda, S., Takeuchi, Y., Fujita, T., Fukumoto, S., and Yamashita, T. 2001. Cloning and characterization of FGF23 as a causative factor of tumor- induced osteomalacia. *Proc Natl Acad Sci U S A* 98:6500-6505.
14. White, K.E., Jonsson, K.B., Carn, G., Hampson, G., Spector, T.D., Mannstadt, M., Lorenz-Depiereux, B., Miyauchi, A., Yang, I.M., Ljunggren, O., et al. 2001. The autosomal dominant hypophosphatemic rickets (ADHR) gene is a secreted polypeptide overexpressed by tumors that cause phosphate wasting. *J Clin Endocrinol Metab* 86:497-500.
15. Larsson, T., Zahradnik, R., Lavigne, J., Ljunggren, O., Juppner, H., and Jonsson, K.B. 2003. Immunohistochemical detection of FGF-23 protein in tumors that cause oncogenic osteomalacia. *Eur J Endocrinol* 148:269-276.
16. Saito, H., Kusano, K., Kinoshita, M., Ito, H., Hirata, M., Segawa, H., Miyamoto, K.I., and Fukushima, N. 2002. Human fibroblast growth factor-23 mutants suppress Na⁺-dependent phosphate Co-transport activity and 1 α ,25-dihydroxyvitamin D₃ production. *J Biol Chem* 277:4144-4150.
17. Shimada, T., Muto, T., Urakawa, I., Yoneya, T., Yamazaki, Y., Okawa, K., Takeuchi, Y., Fujita, T., Fukumoto, S., and Yamashita, T. 2002. Mutant FGF-23

- responsible for autosomal dominant hypophosphatemic rickets is resistant to proteolytic cleavage and causes hypophosphatemia in vivo. *Endocrinology* 143:3179-3182.
18. Bai, X., Miao, D, Goltzman, D, Karaplis, AC. 2003. The autosomal dominant hypophosphatemic rickets R176Q mutation in FGF-23 resists proteolytic cleavage and enhances in vivo biological potency. *J. Biol. Chem. on-line*.
 19. Yamazaki, Y., Okazaki, R., Shibata, M., Hasegawa, Y., Satoh, K., Tajima, T., Takeuchi, Y., Fujita, T., Nakahara, K., Yamashita, T., et al. 2002. Increased circulatory level of biologically active full-length FGF-23 in patients with hypophosphatemic rickets/osteomalacia. *J Clin Endocrinol Metab* 87:4957-4960.
 20. Jonsson, K.B., Zahradnik, R., Larsson, T., White, K.E., Sugimoto, T., Imanishi, Y., Yamamoto, T., Hampson, G., Koshiyama, H., Ljunggren, O., et al. 2003. Fibroblast growth factor 23 in oncogenic osteomalacia and X-linked hypophosphatemia. *N Engl J Med* 348:1656-1663.
 21. Jan de Beur, S.M., Finnegan, R.B., Vassiliadis, J., Cook, B., Barberio, D., Estes, S., Manavalan, P., Petroziello, J., Madden, S.L., Cho, J.Y., et al. 2002. Tumors associated with oncogenic osteomalacia express genes important in bone and mineral metabolism. *J Bone Miner Res* 17:1102-1110.
 22. Ausubel, F. 2003. *Current Protocols in Molecular Biology*: John Wiley & Sons.
 23. Sambrook, J., and Russell, D.W. 2001. *Molecular Cloning, A Laboratory Manual*: Cold Spring Harbor Laboratory Press.
 24. Chen, P., Toribara, T., Warnner H. 1956. Microdetermination of phosphorus. *Anal Chem* 28:1756-1758.
 25. Führ J, K.J., Krüttgen CD. 1955. Eine einfache colorimetrische methode zur inulinbestimmung für nieren-clearance-untersuhngen bei stoffwechselgesunden und diabetikern. *Klin Wochenschr* 33:729-730.
 26. Nagubandi, S., Kumar, R., Londowski, J.M., Corradino, R.A., and Tietz, P.S. 1980. Role of vitamin D glucosiduronate in calcium homeostasis. *J Clin Invest* 66:1274-1280.
 27. Nagubandi, S., Londowski, J.M., Bollman, S., Tietz, P., and Kumar, R. 1981. Synthesis and biological activity of vitamin D3 3 beta-sulfate. Role of vitamin D3 sulfates in calcium homeostasis. *J Biol Chem* 256:5536-5539.
 28. Kumar, R., Nagubandi, S., Jardine, I., Londowski, J.M., and Bollman, S. 1981. The isolation and identification of 5,6-trans-25-hydroxyvitamin D3 from the plasma of rats dosed with vitamin D3. Evidence for a novel mechanism in the metabolism of vitamin D3. *J Biol Chem* 256:9389-9392.
 29. Kumar, R., Londowski, J.M., Murari, M.P., and Nagubandi, S. 1982. Synthesis and biological activity of vitamin D2 3 beta-glucosiduronate and vitamin D2 3 beta-sulfate: role of vitamin D2 conjugates in calcium homeostasis. *J Steroid Biochem* 17:495-502.
 30. Coligan, J., Kruisbeek, AM, Margulies, DM, Shevach, EM, Strober, W. 2003. *Current Protocols in Immunolgy*: John Woley and Sons.
 31. Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227:680-685.
 32. Towbin, H., Staehelin, T., and Gordon, J. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc Natl Acad Sci U S A* 76:4350-4354.
 33. White, K.E., Jonsson, K.B., Carn, G., Hampson, G., Spector, T.D., Mannstadt, M., Lorenz-Depiereux, B., Miyauchi, A., Yang, I.M., Ljunggren, O., et al. 2001. The autosomal dominant hypophosphatemic rickets (ADHR) gene is a secreted polypeptide overexpressed by tumors that cause phosphate wasting. *J Clin Endocrinol Metab* 86:497-500.
 34. Drezner, M.K. 2000. PHEX gene and hypophosphatemia. *Kidney Int* 57:9-18.

35. Yamashita, T., Konishi, M., Miyake, A., Inui, K., and Itoh, N. 2002. Fibroblast growth factor (FGF)-23 inhibits renal phosphate reabsorption by activation of the mitogen-activated protein kinase pathway. *J Biol Chem* 277:28265-28270.
36. 1995. A gene (PEX) with homologies to endopeptidases is mutated in patients with X-linked hypophosphatemic rickets. The HYP Consortium. *Nat Genet* 11:130-136.
37. Shimada, T., Yoneya T., Hino R., Takeuchi Y., Fukumota S., Yamashita T. 2001. Transgenic mice expressing fibroblast growth factor 23 (FGF23) demonstrate hypophosphatemia with low 1,25-dihydroxyvitamin D and rickets/osteomalacia. *J Bone Miner Res* 16 Suppl 1:S151.
38. Shimada, T., Kakitani M., Hasegawa H., Yamazaki Y., Ohguma A., Takeuchi Y., Ujita T., Fukumota S., Tomizuka K., and Yamashita Y. 2002. Targeted ablation of FGF-23 causes hyperphosphatemia, increased 1,25-dihydroxyvitamin D Levels and severe growth retardation. *J Bone Miner Res* 17 Suppl 1:S168.
39. 2000. Autosomal dominant hypophosphatemic rickets is associated with mutations in FGF23. The ADHR Consortium. *Nat Genet* 26:345-348.
40. White, K.E., Cam, G., Lorenz-Depiereux, B., Benet-Pages, A., Strom, T.M., and Econs, M.J. 2001. Autosomal-dominant hypophosphatemic rickets (ADHR) mutations stabilize FGF-23. *Kidney Int* 60:2079-2086.
41. Velculescu, V.E., Zhang, L., Vogelstein, B., and Kinzler, K.W. 1995. Serial analysis of gene expression. *Science* 270:484-487.
42. Velculescu, V.E., Vogelstein, B., and Kinzler, K.W. 2000. Analysing uncharted transcriptomes with SAGE. *Trends Genet* 16:423-425.
43. Berndt, T.J., and Knox, F.G. 1985. Nephron site of resistance to phosphaturic effect of PTH during respiratory alkalosis. *Am J Physiol* 249:F919-922.
44. Berndt, T.J., and Knox, F.G. 1984. Proximal tubule site of inhibition of phosphate reabsorption by calcitonin. *Am J Physiol* 246:F927-930.
45. Tanaka, Y., and Deluca, H.F. 1973. The control of 25-hydroxyvitamin D metabolism by inorganic phosphorus. *Arch Biochem Biophys* 154:566-574.
46. Tanaka, Y., and Deluca, H.F. 1974. Role of 1,25-dihydroxyvitamin D3 in maintaining serum phosphorus and curing rickets. *Proc Natl Acad Sci U S A* 71:1040-1044.
47. Tanaka, Y., Frank, H., and DeLuca, H.F. 1973. Intestinal calcium transport: stimulation by low phosphorus diets. *Science* 181:564-566.
48. Tanaka, Y., Frank, H., and DeLuca, H.F. 1973. Biological activity of 1,25-dihydroxyvitamin D3 in the rat. *Endocrinology* 92:417-422.
49. DeLuca, H.F., and Schnoes, H.K. 1976. Metabolism and mechanism of action of vitamin D. *Annu Rev Biochem* 45:631-666.
50. DeLuca, H.F., and Schnoes, H.K. 1983. Vitamin D: recent advances. *Annu Rev Biochem* 52:411-439.
51. Drezner, M.K. 1984. The role of abnormal vitamin D metabolism in X-linked hypophosphatemic rickets and osteomalacia. *Adv Exp Med Biol* 178:399-404.
52. Biber, J., Hernando, N., Traebert, M., Volkl, H., and Murer, H. 2000. Parathyroid hormone-mediated regulation of renal phosphate reabsorption. *Nephrol Dial Transplant* 15:29-30.
53. Biber, J., Murer, H., and Forster, I. 1998. The renal type II Na⁺/phosphate cotransporter. *J Bioenerg Biomembr* 30:187-194.
54. Karim-Jimenez, Z., Hernando, N., Biber, J., and Murer, H. 2001. Molecular determinants for apical expression of the renal type IIa Na⁺/Pi-cotransporter. *Pflugers Arch* 442:782-790.
55. Rattner, A., Hsieh, J.C., Smallwood, P.M., Gilbert, D.J., Copeland, N.G., Jenkins, N.A., and Nathans, J. 1997. A family of secreted proteins contains homology to the cysteine-rich ligand-binding domain of frizzled receptors. *Proc Natl Acad Sci U S A* 94:2859-2863.

56. Hoang, B., Moos, M., Jr., Vukicevic, S., and Luyten, F.P. 1996. Primary structure and tissue distribution of FRZB, a novel protein related to *Drosophila* frizzled, suggest a role in skeletal morphogenesis. *J Biol Chem* 271:26131-26137.
57. Zorn, A.M. 1997. Cell-cell signalling: frog frizbees. *Curr Biol* 7:R501-504.
58. Jones, S.E., and Jomary, C. 2002. Secreted Frizzled-related proteins: searching for relationships and patterns. *Bioessays* 24:811-820.
59. Dann, C.E., Hsieh, J.C., Rattner, A., Sharma, D., Nathans, J., and Leahy, D.J. 2001. Insights into Wnt binding and signalling from the structures of two Frizzled cysteine-rich domains. *Nature* 412:86-90.
60. Leyns, L., Bouwmeester, T., Kim, S.H., Piccolo, S., and De Robertis, E.M. 1997. Frzb-1 is a secreted antagonist of Wnt signaling expressed in the Spemann organizer. *Cell* 88:747-756.
61. Wang, S., Krinks, M., Lin, K., Luyten, F.P., and Moos, M., Jr. 1997. Frzb, a secreted protein expressed in the Spemann organizer, binds and inhibits Wnt-8. *Cell* 88:757-766.
62. Melkonyan, H.S., Chang, W.C., Shapiro, J.P., Mahadevappa, M., Fitzpatrick, P.A., Kiefer, M.C., Tomei, L.D., and Umansky, S.R. 1997. SARPs: a family of secreted apoptosis-related proteins. *Proc Natl Acad Sci U S A* 94:13636-13641.
63. Mayr, T., Deutsch, U., Kuhl, M., Drexler, H.C., Lottspeich, F., Deutzmann, R., Wedlich, D., and Risau, W. 1997. Fritz: a secreted frizzled-related protein that inhibits Wnt activity. *Mech Dev* 63:109-125.
64. Hoang, B.H., Thomas, J.T., Abdul-Karim, F.W., Correia, K.M., Conlon, R.A., Luyten, F.P., and Ballock, R.T. 1998. Expression pattern of two Frizzled-related genes, Frzb-1 and Sfrp-1, during mouse embryogenesis suggests a role for modulating action of Wnt family members. *Dev Dyn* 212:364-372.
65. Finch, P.W., He, X., Kelley, M.J., Uren, A., Schaudies, R.P., Popescu, N.C., Rudikoff, S., Aaronson, S.A., Varmus, H.E., and Rubin, J.S. 1997. Purification and molecular cloning of a secreted, Frizzled-related antagonist of Wnt action. *Proc Natl Acad Sci U S A* 94:6770-6775.
66. Lin, K., Wang, S., Julius, M.A., Kitajewski, J., Moos, M., Jr., and Luyten, F.P. 1997. The cysteine-rich frizzled domain of Frzb-1 is required and sufficient for modulation of Wnt signaling. *Proc Natl Acad Sci U S A* 94:11196-11200.
67. Bafico, A., Gazit, A., Pramila, T., Finch, P.W., Yaniv, A., and Aaronson, S.A. 1999. Interaction of frizzled related protein (FRP) with Wnt ligands and the frizzled receptor suggests alternative mechanisms for FRP inhibition of Wnt signaling. *J Biol Chem* 274:16180-16187.
68. Hsieh, J.C., Rattner, A., Smallwood, P.M., and Nathans, J. 1999. Biochemical characterization of Wnt-frizzled interactions using a soluble, biologically active vertebrate Wnt protein. *Proc Natl Acad Sci U S A* 96:3546-3551.
69. Nusse, R. 1992. The Wnt gene family in tumorigenesis and in normal development. *J Steroid Biochem Mol Biol* 43:9-12.
70. Nguyen HT, T.A., Kogan BA, Baskin LS, and Cunha GR. 1999. Expression of the Wnt gene family during late nephrogenesis and complete ureteral obstruction. *Lab Invest* 79(6):647.
71. Nusse, R., and Varmus, H.E. 1992. Wnt genes. *Cell* 69:1073-1087.
72. Vainio, S.J., and Uusitalo, M.S. 2000. A road to kidney tubules via the Wnt pathway. *Pediatr Nephrol* 15:151-156.
73. Vainio, S.J., Itaranta, P.V., Perasaari, J.P., and Uusitalo, M.S. 1999. Wnts as kidney tubule inducing factors. *Int J Dev Biol* 43:419-423.
74. Sakanaka, C., Sun, T.Q., and Williams, L.T. 2000. New steps in the Wnt/beta-catenin signal transduction pathway. *Recent Prog Horm Res* 55:225-236.
75. Uusitalo, M., Heikkila, M., and Vainio, S. 1999. Molecular genetic studies of Wnt signaling in the mouse. *Exp Cell Res* 253:336-348.

76. Povelones, M., and Nusse, R. 2002. Wnt signalling sees spots. *Nat Cell Biol* 4:E249-250.
77. Pandur, P., Lasche, M., Eisenberg, L.M., and Kuhl, M. 2002. Wnt-11 activation of a non-canonical Wnt signalling pathway is required for cardiogenesis. *Nature* 418:636-641.
78. Pandur, P., Maurus, D., and Kuhl, M. 2002. Increasingly complex: new players enter the Wnt signaling network. *Bioessays* 24:881-884.
79. Rattner, A., Hsieh J-C, Smallwood PM, Gilbert DJ, Copeland NG, Jenkins NA, and Nathans J. 1997. A family of secreted proteins contains homology to the cysteine-rich ligand-binding domain of frizzled receptors. *Proc. Natl. Acad. Sci. USA* 94:2859-2863.
80. Wolf, V., Ke, G., Dharmarajan, A.M., Bielke, W., Artuso, L., Saurer, S., and Friis, R. 1997. DDC-4, an apoptosis-associated gene, is a secreted frizzled relative. *FEBS Lett* 417:385-389.
81. Bejsovec, A. 2000. Wnt signaling: An embarrassment of receptors. *Curr Biol* 10:R919-R922.
82. Cadigan, K.M., and Nusse, R. 1997. Wnt signaling: a common theme in animal development. *Genes Dev* 11:3286-3305.
83. Dale, T.C. 1998. Signal transduction by the Wnt family of ligands. *Biochem J* 329:209-223.
84. James, I.E., Kumar, S., Barnes, M.R., Gress, C.J., Hand, A.T., Dodds, R.A., Connor, J.R., Bradley, B.R., Campbell, D.A., Grabill, S.E., et al. 2000. FrzB-2: a human secreted frizzled-related protein with a potential role in chondrocyte apoptosis. *Osteoarthritis Cartilage* 8:452-463.
85. Schumann, H., Holtz, J., Zerkowski, H.R., and Hatzfeld, M. 2000. Expression of secreted frizzled related proteins 3 and 4 in human ventricular myocardium correlates with apoptosis related gene expression. *Cardiovasc Res* 45:720-728.
86. Fujita, M., Ogawa, S., Fukuoka, H., Tsukui, T., Nemoto, N., Tsutsumi, O., Ouchi, Y., and Inoue, S. 2002. Differential expression of secreted frizzled-related protein 4 in decidual cells during pregnancy. *J Mol Endocrinol* 28:213-223.
87. Wong, S.C., Lo, S.F., Lee, K.C., Yam, J.W., Chan, J.K., and Wendy Hsiao, W.L. 2002. Expression of frizzled-related protein and Wnt-signalling molecules in invasive human breast tumours. *J Pathol* 196:145-153.
88. Leimeister, C., Bach, A., and Gessler, M. 1998. Developmental expression patterns of mouse sFRP genes encoding members of the secreted frizzled related protein family. *Mech Dev* 75:29-42.



CP1102302B-4

FIGURE 1

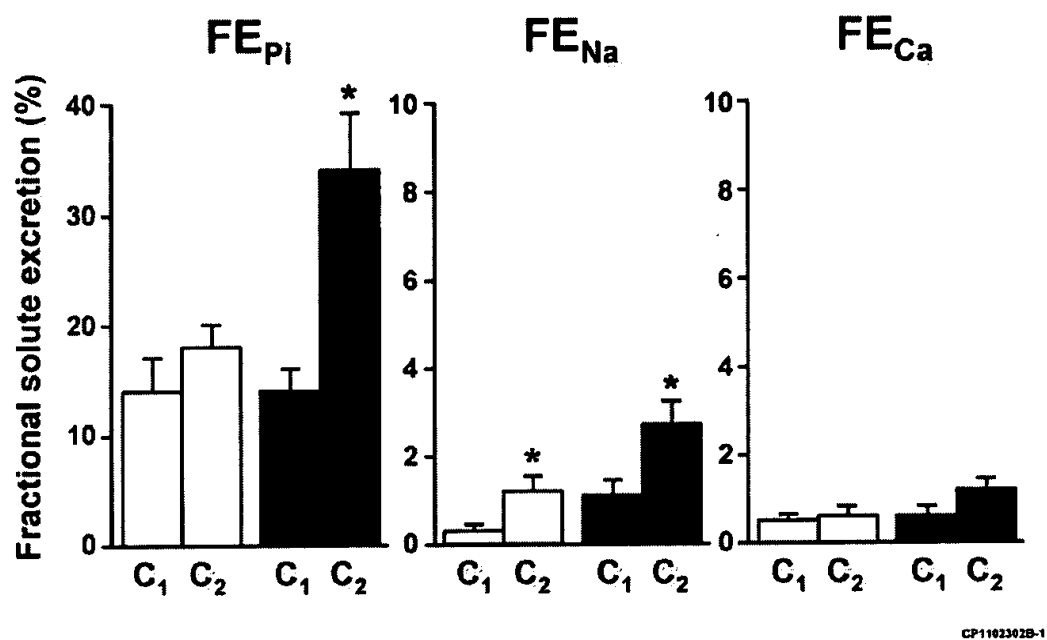
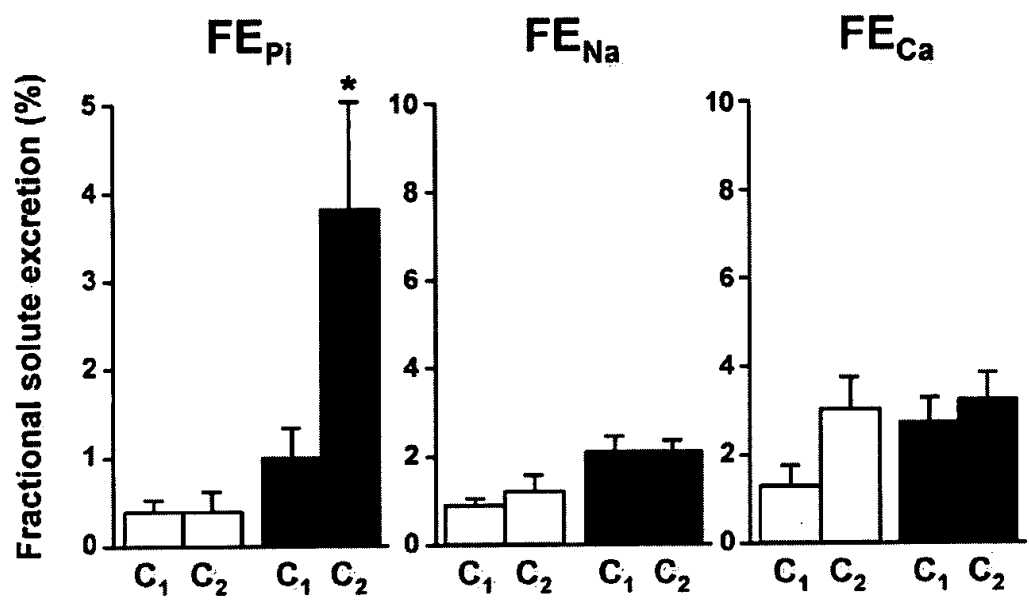


FIGURE 2



CP1102302B-2

FIGURE 3

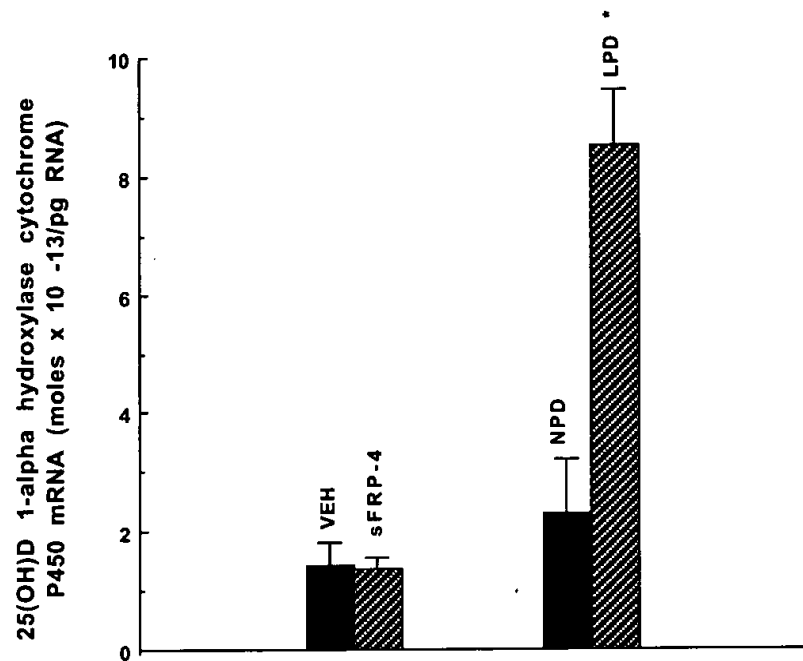


Figure 4

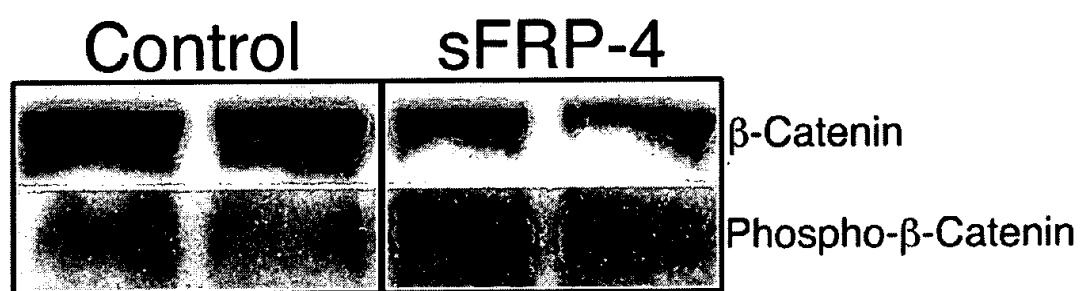


FIGURE 5

the SFRP family that contains a cysteine-rich domain homologous to the putative Wnt-binding site of Frizzled proteins. SFRPs act as soluble modulators of Wnt signaling. The expression of SFRP4 in ventricular myocardium correlates with apoptosis related gene expression.

```

FEATURES             Location/Qualifiers
    source             1..2820
                        /organism="Homo sapiens"
                        /mol_type="mRNA"
                        /db_xref="taxon:9606"
                        /chromosome="7"
                        /map="7p14.1"
    gene               1..2820
                        /gene="SFRP4"
                        /note="synonyms: FRP -4, FRPHE"
                        /db_xref="LocusID: 6424"
                        /db_xref="MIM: 606570"
    CDS                238..1278
                        /gene="SFRP4"
                        /codon_start=1
                        /product="secreted frizzled-related protein 4"
                        /protein_id="NP_003005.1"
                        /db_xref="GI:4506895"
                        /db_xref="LocusID: 6424"
                        /db_xref="MIM: 606570"
                        /translation="MFLSIL VALCLWLHLALGVRGAPCEAVRIPMCRHMPWNITRMPN
HLHHSTQENAILAIEQYEEL VDVNCSAVLRFFFCAMYAPICTLEFLHDPIKPKKSVCQ
RARDDCEPLMKMYNHSWPES LACDELPVYDRGVCISPEAIVTDLPEDVKWIDITPDM
VQERPLDVDCRKLSPDRCKC KVKPTLATYLSKNYSYVIHAKIKAVQRSGCNEVTTVV
DVKEIFKSSSPIPTQVPLI TNSSCQCPHILPHQDVLIMCYEWSRMMLLENCLVEKW
RDQLSKRSIQWEERLQEQRR TVQDKKKTAGRTSRSNPPKPKGKPPAPKPASPKNKI
RSAQKRTNPKRV"
    misc_feature       304..657
                        /gene="SFRP4"
                        /note="FRI; Region: Frizzled"
                        /db_xref="CDD: smart00063"
    misc_feature       796..1116
                        /gene="SFRP4"
                        /note="NTR; Region: NTR/C345C module. We have not included
the related pfam00965 family. It has been suggested that
the common function of these modules is binding to
metzincins. A subset of this family is known as the C345C
domain because it occurs in complement C3, C4 and C5"
                        /db_xref="CDD: pfam01759"
    polyA_signal       2799..2804
                        /gene="SFRP4"

```

```

BASE COUNT      803 a      619 c      643 g      755 t
ORIGIN

```

```

1  ggcgggttcg cgccccgaag gctgagagc t ggcgctgctc gtgccctgtg tgccagacgg
61  cggagctccg cggccggacc ccgcgggccc c gctttgctgc cgactggagt ttgggggaag
121 aaactctcct gcgccccaga agatttctt c ctcggcgaag ggacagcgaa agatgagggg
181 ggcaggaaga gaaggcgctt tctgtctgc c ggggtcgcag cgcgagaggg cagtgccatg
241 ttcctctcca tctagtggc gctgtgcct g tggctgcacc tggcgctggg cgtgcgcggc
301 gcgccttgcg aggcggtgcg catccctat g tgccggcaca tgccctggaa catcacgcgg
361 atgccccacc acctgcacca cagcacgca g gagaacgcca tcctggccat cgagcagtac
421 gaggagctgg tggacgtgaa ctgcagcgc c gtgctgcgct tcttcttctg tgccatgtac
481 gcgcccattt gcaccctgga gttcctgca c gaccctatca agccgtgcaa gtcggtgtgc
541 caacgcgcgc gcgacgactg cgagcccct c atgaagatgt acaaccacag ctggcccgaa
601 agcctggcct gcgacgagct gcctgtcta t gaccgtggcg tgtgcatttc gcctgaagcc
661 atcgtcacgg acctcccga g gatgttaa g tggatagaca tcacaccaga catgatgga

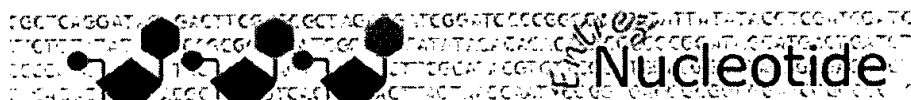
```

721 caggaaaggc ctcttgatgt tgactgtaa a cgcctaagcc ccgatacggg caagtgtaaa
781 aagggtgaagc caactttggc aacgtatct c agcaaaaact acagctatgt tattcatgcc
841 aaaataaaaag ctgtgcagag gagtggctg c aatgaggtca caacgggtgg ggatgtaaaa
901 gagatcttca agtcctcatc acccatccc t cgaactcaag tcccgtcat tacaattct
961 tcttgccagt gtccacacat cctgcccc t caagatgtt tcatcatgtg ttacgagtgg
1021 cgttcaagga tgatgcttct tgaaaattg c ttagttgaaa aatggagaga tcagcttagt
1081 aaaagatcca tacagtggga agagaggct g caggaacagc ggagaacagt tcaggacaag
1141 aagaaaacag ccgggcgcac cagtcgtag t aatccccca aaccaaaggg aaagcctcct
1201 gctcccaaac cagccagtcc caagaagaa c attaaaacta ggagtgccca gaagagaaca
1261 aaccgaaaaa gagtgtgagc taactagtt t ccaaagcgga gacttccgac ttcttacag
1321 gatgaggctg ggcattgcct gggacagcc t atgtaaggcc atgtgccct tgcctaaca
1381 actcactgca gtgctcttca tagacacat c ttgcagcatt tttcttaagg ctatgcttca
1441 gtttttcttt gtaagccatc acaagccat a gtggtaggtt tgccttttgg tacagaaggt
1501 gagttaaagc tgggtgaaaa ggcttattg c attgcattca gagtaacctg tgtgcatact
1561 ctagaagagt agggaaaata atgcttgtt a caattcgacc taatatgtgc attgtaaaat
1621 aaatgccata tttcaaaca aacacgtaa t ttttttacag tatgttttat taccttttga
1681 tatctgttgt tgcaatgtta gtgatgttt t aaaatgtgat gaaaatataa tgtttttaag
1741 aaggaacagt agtggaatga atgttaaaa g atctttatgt gtttatggc tcgagaagga
1801 tttttgtgat gaaagggat tttttgaaa a attagagaag tagcatatgg aaaattataa
1861 tgtgtttttt taccaatgac ttcagtttc t gtttttagct agaaacttaa aaacaaaaat
1921 aataataaag aaaaataaat aaaaaggag a ggcagacaat gtctggattc ctgttttttg
1981 gttacctgat ttccatgatc atgatgctt c ttgtcaacac cctcttaagc agcaccagaa
2041 acagtgaagt tgtctgtacc attaggagt t aggtactaat tagttggcta atgctcaagt
2101 attttatacc cacaagagag gtatgtcac t catcttactt ccaggacat ccacctgag
2161 aataatttga caagcttaaa aatggcctt c atgtgagtgc caaattttgt tttcttcat
2221 ttaaatattt tctttgccta aatacatgt g agaggagtta aatataaatg tacagagagg
2281 aaagttgagt tccacctctg aaatgagaa t tacttgacag ttgggatact ttaatcagaa
2341 aaaaagaact tatttgcagc attttatca a caaatttcat aattgtggac aattggaggc
2401 atttatttta aaaaacaatt ttattggcc t tttgctaaca cagtaagcat gtattttata
2461 aggcattcaa taaatgcaca acgcccaa g gaaataaaat cctatcta cctactctcc
2521 actacacaga ggtaatcact attagtatt t tggcatatta ttctccagg gtttgcttat
2581 gcacttataa aatgatttga acaaataaa a ctaggaacct gtatacatgt gtttcataac
2641 ctgcctcctt tgcttggccc tttattgag a taagttttcc tgtcaagaaa gcagaaacca
2701 tctcatttct aacagctgtg ttatatcc a tagtatgcat tactcaacaa actgttgtgc
2761 tattggatac ttaggtggtt tcttcactg a caatactgaa taaacatctc accggaattc

//

[Disclaimer](#) | [Write to the Help Desk](#)
[NCBI](#) | [NLM](#) | [NIH](#)

May 1 2003 16:27:42



PubMed

Nucleotide

Protein

Genome

Structure

PMC

Taxonomy

OMIM

Bio

Search

for

Limits

Preview/Index

History

Clipboard

Details

Display

Show:

Send to

☐ 1: NM_016687. Mus musculus secr...[gi:7710093]

Links

LOCUS Sfrp4 1785 bp mRNA linear ROD 08-APR-2003
 DEFINITION Mus musculus secreted frizzled-related sequence protein 4 (Sfrp4), mRNA.

ACCESSION NM_016687

VERSION NM_016687.1 GI:7710093

KEYWORDS .

SOURCE Mus musculus (house mouse)

ORGANISM Mus musculus

Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi;
 Mammalia; Eutheria; Rodentia; Sciurognathi; Muridae; Murinae; Mus.

REFERENCE 1 (bases 1 to 1785)

AUTHORS Chang, J.T., Esumi, N., Moore, K., Li, Y., Zhang, S., Chew, C.,
 Goodman, B., Rattner, A., Moody, S., Stetten, G., Campochiaro, P.A. and
 Zack, D.J.

TITLE Cloning and characterization of a secreted frizzled-related protein
 that is expressed by the retinal pigment epithelium

JOURNAL Hum. Mol. Genet. (1999)

REFERENCE 2 (bases 1 to 1785)

AUTHORS Rattner, A., Hsieh, J.C., Smallwood, P.M., Gilbert, D.J.,
 Copeland, N.G., Jenkins, N.A. and Nathans, J.

TITLE A family of secreted proteins contains homology to the
 cysteine-rich ligand-binding domain of frizzled receptors

JOURNAL Proc. Natl. Acad. Sci. U.S.A. 94 (7), 2859-2863 (1997)

MEDLINE 97250455

PUBMED 9096311

COMMENT PROVISIONAL REFSEQ: This record has not yet been subject to final
 NCBI review. The reference sequence was derived from AF117709.1.

FEATURES Location/Qualifiers

source

1..1785

/organism="Mus musculus"

/mol_type="mRNA"

/db_xref="taxon:10090"

/chromosome="13"

/map="13 7.0 cM"

gene

1..1785

/gene="Sfrp4"

/db_xref="LocusID:20379"

/db_xref="MGI:892010"

CDS

274..1329

/gene="Sfrp4"

/codon_start=1

/product="secreted frizzled-related sequence protein 4"

/protein_id="NP_057896.1"

/db_xref="GI:7710094"

/db_xref="LocusID:20379"

/db_xref="MGI:892010"

/translation="MLRSILVALCLWLRLALGVRGAPCEAVRI PMCRHMPWNITRMPN"

HLHHSTQENAILAIEQYEELVDVNCSSVLRFFLCAMYAPICTLEFLHDPIKPKKSVCQ
RARDCEPLMKMYNHSWPESLACDELPVYDRGVCISPEAIVTDLPEDVKWIDITPDM
VQERSFDADCKRLSPDRCKCKVKPTLATYLSKNYSYVIHAKIKAVQRSGCNEVTTVV
DVKEIFKSLSPIPRTQVPLITNSSCQCPHILPHQDVLIMCYEWRSRMMLLENCLVEKW
RDQLSRRSIQWEERLQEQQRTIQDKKQIASRTSRTSRSNPPKSKGRPPAPKPASPKN
IKARSAPKKS NLKKSAS"

misc feature

340..693
/gene="Sfrp4"
/note="FRI; Region: Frizzled"
/db_xref="CDD:smart00063"

misc feature

832..1125
/gene="Sfrp4"
/note="NTR; Region: NTR/C345C module. We have not included
the related pfam00965 family. It has been suggested that
the common function of these modules is binding to
metzincins. A subset of this family is known as the C345C
domain because it occurs in complement C3, C4 and C5"
/db_xref="CDD:pfam01759"

BASE COUNT 478 a 454 c 453 g 399 t 1 others
ORIGIN

```
1  ccacgcgtcc  gcggactcgt  gggccgagct  gcgggactcg  agactggagc  ttcccgcggg
61  ggtcgttgct  ctggaggctg  agtgctggcg  ctgccagtgc  cgggtgcgcc  aggggtgatc
121  gacggccaac  cccccagaac  tcctcatcag  tgcagactgg  agctccagca  ggaaagctca
181  ctccactgcc  cctaggtaaa  gagataagga  ggtcaacggg  gctggaagaa  aagggtcctc
241  ccagcagaga  ctgcagccca  ggaggacagt  gccatgctcc  gctccatcct  ggtggcggtt
301  tgctgtggc  tgcgcctggc  gctgggagtt  cgcgagcgc  cctgcgaagc  tgtgcgcctc
361  cccatgtgca  ggcacatgcc  ctggaacatc  acccgatgc  ccaaccacct  gcaccacagc
421  actcaggaga  acgccatcct  ggccatcgaa  cagtatgaag  agctagtggg  cgtgaactgc
481  agctctgtgc  tgcgcttctt  cctttgtgcc  atgtacgcac  ccatctgtac  cctggagttc
541  ctgcacgatc  ccatcaagcc  ctgcaagtct  gtgtgccagc  gcgcacgtga  cgactgcgag
601  cccctcatga  agatgtataa  ccacagctgg  ccagagagcc  tggcctgcga  tgagctgccg
661  gtctatgacc  gtggagtttg  tatctctcca  gaggcaatag  tcactgacct  tccagaagat
721  gtgaagtggg  tagacatcac  accagatatg  atggtgcaag  aaaggctcct  tgatgctgac
781  tgtaaacgtc  tgagccctga  tcggtgcaag  tgcaaaaagg  tgaagccgac  cctggcaaca
841  tacctgagca  aaaactacag  ctatgttatt  catgccaaaa  taaaagccgt  ccagaggagt
901  ggttgcaatg  aggtcacaac  tgtggtagat  gtaaaagaga  tcttcaagtc  tttgtcacct
961  atccctcgaa  cacaagtcct  tctcatcacc  aattcctcct  gccagtgtcc  acatatcctg
1021  ccccatcaag  atgtcctaag  catgtgttat  gagtggcggt  caaggatgat  gcttcttgaa
1081  aattgcttag  ttgaaaagtg  gagagatcaa  ctcagtagaa  ggtccataca  gtgggaagag
1141  aggttcagg  aacagcagag  aacaattcag  gacaagaagc  agatagccag  ccgcaccagt
1201  cgcaccagtc  gcagtaacct  cccaaagtca  aagggaaggc  cacctgctcc  caaacctgcc
1261  agtcctaaga  agaacatcaa  agctagaagt  gccccaaaa  agtcaaacct  gaagaaaagc
1321  gcaagctaag  tactttctgt  gatggagcca  cccttacagg  ataagggtgt  gctgcctggg
1381  acagcctaca  gaaggccaca  taccacttgc  ctaaggacc  cactgtgggt  ctctttatag
1441  acacttcttg  cagcatgtct  cgtaatgata  gcttcagtgt  tttgctttta  gccacagagc
1501  cctgttactt  gttggttttc  tgggtatggg  agtacaagag  ggaaggcttg  tggaaagggt
1561  cctgaattca  cagttgtctg  tgtgcatact  gtgtaagacc  tcctgtttta  agagtttgac
1621  ctaaaatgta  cattataaag  ttacatact  atgtcaagca  aaactttttg  acaaaaaata
1681  ttggatatcc  tttgacttgt  gttaagaagt  tgggtgatata  ttaaatgtta  ttgaaaacat
1741  gtttttgata  aaggcagggg  aatgatata  tccanaaaa  aaaaa
```

//

[Disclaimer](#) | [Write to the Help Desk](#)
[NCBI](#) | [NLM](#) | [NIH](#)

Jun 19 2003 12:37 45

Nucleotide

Boo

Details

Get Subsequence

Links

ORGANISM *Rattus norvegicus*

Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi;
Mammalia; Eutheria; Rodentia; Sciurognathi; Muridae; Murinae;
Rattus.

AUTHORS Fujita, M., Ogawa, S., Fukuoka, H., Tsukui, T., Nemoto, N., Tsutsumi, O.,
Ouchi, Y. and Inoue, S.

TITLE Differential expression of secreted frizzled-related protein 4 in decidua cells during pregnancy

JOURNAL J. Mol. Endocrinol. 28 (3), 213-223 (2002)

MEDLINE 22058279

PUBMED 12063187

REMARK GeneRIF: Differential expression of secreted frizzled-related protein 4 in decidual cells during pregnancy.

REFERENCE 2 (bases 1 to 1910)

AUTHORS Yam, J.W., Chan, K.W., Wong, V.K. and Hsiao, W.L.

TITLE Transcriptional activity of the promoter region of rat
frizzled-related protein gene

JOURNAL Biochem. Biophys. Res. Commun. 286 (1), 94-100 (2001)

MEDLINE 21378144

PUBMED	<u>11485313</u>
--------	-----------------

REFERENCE 3 (bases 1 to 1910)

AUTHORS Wolf, V., Artuso, L., Dharmarajan, A., Guo, K., Bielke, W. and Friis, R.R.

TITLE A frizzled related Gene is upregulated in Physiological Apoptosis

JOURNAL Unpublished

REFERENCE 4 (bases 1 to 1910)

AUTHORS Guo,K., Wolf,V., Dharmarajan,A., Feng,Z., Bielke,W., Susanne,S. and Friis,R.R.

TITLE Apoptosis-associated Gene Expression in Corpus luteum of the Rat

JOURNAL Unpublished

COMMENT PROVISIONAL REFSEQ: This record has not yet been subject to final
NCBI review. The reference sequence was derived from AF012891.1.

FEATURES	Location/Qualifiers
----------	---------------------

source

1..1910

```
/organism="Rattus norvegicus"
```

```
/mol type="mRNA"
```

```
/strain="Sprague-Dawley"
```

```
/db xref="taxon:10116"
```

```
/chromosome="17"
```

/map="17q12.1"
 gene 1..1910
 /gene="Sfrp4"
 /db_xref="LocusID:89803"
 /db_xref="RGD:621075"
 CDS 95..1141
 /gene="Sfrp4"
 /codon_start=1
 /product="secreted frizzled-related protein 4"
 /protein_id="NP_445996.1"
 /db_xref="GI:16758312"
 /db_xref="LocusID:89803"
 /db_xref="RGD:621075"
 /translation="MLLSILVALCLCVRLALGVRGAPCEAVRIPMCRHMPWNITRMPN
 HLHHSTQENAILAIEQYEELVDVNCSSVLRFFLCAMYAPICTLEFLHDPFKPKCSVCQ
 RARDDCEPLMKMYNHSWPESLACDELPHYDRGVCISPEAIVTDLPEDVKWIDITPDMM
 VQERSFDADCKHLSFDRCKCKVKPTLATYLSKNYSYVIHAKIKAVQRSGCNEVTTVV
 DVKEIFKSSSPIRPTQVPLITNSSCQCPHILPXQDVLIMCYERRSRMMLLENCLVEKW
 RDQLSRRSTQWEERLQEQRTTQDKKQIASRTSRSNPPKPKGRSPASKPASPKKNKA
 RSAPKKSNNPKKSTS"
 misc_feature 161..514
 /gene="Sfrp4"
 /note="FRI; Region: Frizzled"
 /db_xref="CDD:smart00063"
 misc_feature 653..946
 /gene="Sfrp4"
 /note="NTR; Region: NTR/C345C module. We have not included
 the related pfam00965 family. It has been suggested that
 the common function of these modules is binding to
 metzincins. A subset of this family is known as the C345C
 domain because it occurs in complement C3, C4 and C5"
 /db_xref="CDD:pfam01759"

BASE COUNT 530 a 458 c 448 g 473 t 1 others
 ORIGIN

```

1 agctcggagc gcacgcccta ggtaaagaca taaggagatc agaggggactt gaaggggaagg
61 tccccagtgg gacgcagccc aggaggacag tgcgatgctc ctctccatcc tggtagcggtt
121 atgcctgtgc gtgcgcctgg ctctgggagt gcgcggagcg ccctgcgagg ctgtgcgcat
181 ccccatgtgt aggcacatgc cctggaacat caccocggatg cccaaccacc tgcaccacag
241 cactcaggag aacgccatcc tggccatcga gcagtacgag gagctggtag acgtgaactg
301 cagctctgta ctgcgcttct ttctctgtgc catgtatgca cccatctgta ccctggagtt
361 cctgcacgat cccatcaaac cctgcaaate tgtgtgccag cgcgcacgcg acgactgcga
421 gcccctcatg aagatgtata accacagctg gccagagagc ctggcttgcg atgagctgcc
481 tgtctatgac cgtggagtgt gcatctctcc ggaggcgata gtcactgacc ttccggaaga
541 tgtgaagtgg atagacatca caccagatat gatggttcaa gaaaggtcct ttgatgctga
601 ctgtaaacat ctgagccctg atcgggtgcaa gtgcaaaaag gtgaagccaa ctttggcaac
661 gtacctgagc aaaaactaca gctatggtat tcatgccaaa ataaaagcgg tccagaggag
721 tggttgcaat gaggtcacaa ctgtggtcga tgtaaaagag atcttcaagt cttcatcacc
781 tatccctcga acgcaagtc cctcatcac caattcctcc tgccagtgtc cacacatcct
841 gcccctcaa gatgtcctaa tcatgtgtta tgagcggcgt tcaaggatga tgcttcttga
901 aaattgttta gttgagaaat ggagagatca actaagcaga aggtccacac agtggaaga
961 gaggttcag gaacagcaga gaacaactca ggacaagaag caaatagcca gccgcaccag
1021 tcgcagtaac ccccaaagc caaagggaag gtcacctgct tccaaacctg ccagtcctaa
1081 gaagaacatc aaagctagaa gtgcacccaa aaagtcaaac ccaaagaaac gatacaagcta
1141 attactttcc aagttgaagc cacccttaca ggataagggtg gtactgcctg ggacagcctt
1201 ccgaaggcca cacaccactt gccttaggac ccactgtggt tctctttata gacatgtctt
1261 gcagcatttc tcttaatgat agcttcagta tttctcttta aaccacagat ccctgttatt
1321 tgtcagtttt cttttatgga aaggtacctg tgttcagagt ggctgtgag catactgtgt
1381 gaccttctgc tttgagattt tgacctaaaa tctaccttat aaagttcaca tactatgtca
1441 agcaaaactt tttttgacaa aaatattgga tctcctttga cttctgttag gaagttagtg
1501 atatataaaa tgttattgaa aaaaatgttt ttgacaaaagg cagaagaatg atacataaaa
  
```

1561 gaccttcatg tgtttgttgt ctgcagaaag agttttctga tgaatgtggt tttcaaaaac
1621 ttgaggtatg ggatgagggg aatgataatg tgggtttgta aaactttctc ttctgtcttt
1681 agttagaaat ttctaaaacg aagccaaaaa tgatgagtaa agggactgtc gatgtttatg
1741 ttcttgctgt ctgattgcca cagcatcctg gattctctc aacagtgacc catgagccac
1801 cccacagaca gtatgcttct tttctcctgt atgaattggg taccaaggag acatcttggt
1861 taatccatca agagagctgg gccatccac tccagggcat cgtaagggaa

//

[Disclaimer](#) | [Write to the Help Desk](#)
[NCBI](#) | [NLM](#) | [NIH](#)

Jun 19 2003 12:37:45